The Impact of Target Site Alterations to Fluoroquinolones in *Streptococcus pneumoniae*

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2001

Abstract

The fluoroquinolones are a class of potent antibacterial agents that have been used in the treatment of infectious diseases for the previous two decades. Fluoroquinolones cause cell death through interactions with Class II topoisomerases. These enzymes are key players in maintaining cellular integrity in bacteria and the disruption of these proteins through mutational events cause severe cellular damage and death. Until recently, the use of fluoroquinolones was limited to the treatment of gram-negative infections, the introduction of extended-spectrum quinolones now allows the use of these compounds against gram-positive pathogens which cause severe respiratory tract infections. Respiratory tract infections are a predominant indication for antibacterial prescribing in both hospital and community acquired respiratory illness.

Initially the antibacterial activity of the extended-spectrum fluoroquinolones was assessed against the three major respiratory pathogens and compared with other similar antibiotics. Prior to this sensitivity study the current MIC testing protocols for the quinolones were evaluated. It was found that carbon dioxide incubation adversely affected the media and antibacterial efficacy of the antibiotic subsequently resulting in elevated MICs for all the fluoroquinolones. This effect was not confined to organisms that required carbon dioxide incubation conditions for growth. Gram-negative pathogens such as *Ps. aeruginosa* also exhibited significant MIC increases in reduced air conditions. The quinolone MICs reported for *S. pneumoniae* were also increased in reduced air conditions in comparison to air. These observations prompted all further sensitivity testing to be performed in ambient air conditions.

The sensitivity survey was done on 909 isolates recovered from nine centres from around the United Kingdom. Sensitivity was determined by testing each isolate for its minimum inhibitory concentration (MIC) by agar dilution. Against all three respiratory pathogens the extended-spectrum fluoroquinolones were found to be the most bactericidal. Ciprofloxacin retained its activity against both *H. influenzae* and

M. catarrhalis, although activity against *S. pneumoniae* was variable. Tetracycline was found to be the least active against all three species.

Thirty-one clinical pneumococcal isolates that exhibited decreased sensitivity to the fluoroquinolones were found to express a combination of resistance mechanisms. Efflux inhibition studies have shown that all the selected pneumococcal isolates demonstrated a reduction in susceptibility to ciprofloxacin and norfloxacin in the presence of reserpine (efflux pump inhibitor). No such effect was observed with either of the extended-spectrum quinolones (moxifloxacin and gatifloxacin) tested. Sequencing analysis of 11 representative isolates revealed that none of the organisms sustained a genetic mutation in either *gyrA* or *par*C except for two strains: 7368, which demonstrated a Lysine 137 → Asparagine change and BPL27, which has a Aspartic acid 83 → Asparagine mutation. Both these strains exhibited a MIC of 4mg/l to ciprofloxacin but remained sensitive to moxifloxacin. Other isolates that exhibited identical MICs were found not to possess these genetic changes.

Previous research has suggested that the primary fluoroquinolone target in gram-negative bacteria is DNA gyrase A and within gram-positive bacteria is topoisomerase IV. *In vitro* mutation studies with anti-gram positive quinolones such as gatifloxacin and sparfloxacin have shown that *gyrA* is the primary intracellular target in *S. pneumoniae*. Therefore to ascertain the primary target of moxifloxacin within *S. pneumoniae*, stepwise selection of four different pneumococcal phenotypes (laboratory strain *S. pneumoniae* R6, penicillin intermediate and resistant clinical isolates 285 and 158 and *pmrA* efflux hyperexpresser P1Z1/IN27) was done. The *in vitro* studies, indicated that the development of moxifloxacin resistance was through a combination of mutations within the target genes and efflux pump expression. There were two pathways of resistance development where the first started with a Serine80 → Tyrosine change accompanied by the apparent induction of an efflux pump. This was followed by a Serine79 → Tyrosine mutation in ParC with the loss of the efflux pump. The second pathway showed no initial change in any of the target genes but was accompanied by the switching on of an efflux pump. No additional mutations were detected in either the *parE* and *gyrB* genes.

Accumulation assays show that uptake is dramatically reduced in mutants after moxifloxacin challenge in comparison to the respective parent strains. The contribution of the efflux pump *pmrA* was analysed in isogenic moxifloxacin mutants. Northern blot analysis demonstrated the expression of a *pmrA* or a *pmrA* like pump in first-step and some second-step mutants indicating that efflux may contribute to moxifloxacin resistance to a greater extent than previously assumed. Therefore, these data suggest that a *pmrA* like pump may contribute to the efflux mediated resistance towards moxifloxacin, although the contribution of this efflux mechanism was not evaluated in clinical isolates.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

T. Dorai-Schneiders

Acknowledgements

I would like to thank my supervisor, Professor S.G.B. Amyes for his excellent supervision and encouragement throughout this project. I would also like to express my thanks to Dr. C. J. Thomson from Bayer Plc for providing the financial support and supervision throughout the project. I would like to thank Bayer Plc for the studentship that funded this project. Thanks must go to all my colleagues and staff of Molecular Chemotherapy. Special thanks must go to my family for encouraging me throughout this time. To Johannes for all the late nights, formatting, support and preserving my sanity throughout. I would like to dedicate this thesis to my Uncle Elango without whose support none of this would have begun and to Johannes who has been a source of support and strength to me.

Presentations

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Abbreviations

ADP	Adenosine di-phosphate
ADPNP	Non hydrolysable ATP analogue
ATP	Adenosine tri-phosphate
bp	base pair
BHI broth	Brain Heart Infusion broth
CBA	Columbia Agar Base
cfu	colony forming units
CO_2	carbon dioxide
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
GABA	Gamma amino-butyric acid
MIC	Minimum Inhibitory Concentration
MOX	Moxifloxacin
MPC	Mutant Prevention Concentration
NSAIDS	Non stereoidal anti-inflammatory drugs
PCR	Polymerase Chain Reaction
QRDR	Quinolone Resistance Determining Region
RTI	Respiratory Tract Infections
RNA	Ribonucleic acid
TAE	Tris-acetate Buffer
TH	Todd Hewitt Broth
w/v	weight per volume
v/v	volume per volume

The standard single letter and three letter abbreviations are used for the amino acids.

Contents

1 Introduction

1.1 Prologue

In 1969, the US Surgeon General testifying to the congress made the historic statement "the time has come to close the book on infectious diseases"[1]. He made these statements at an optimistic time when more than 25,000 antibiotic products had been developed and antibiotic resistance was not a major public health issue. Three decades on, we look to a dwindling resource of treatment options and the prevalence of antibiotic resistance is sufficiently serious to threaten a future, which could date back to the pre-antibiotic era. The widespread use of antibiotics in clinical therapy began with the introduction of the penicillins in the 1940s, the quinolones in 1964, the cephalosporins in 1975, the fluoroquinolones and carbapenems in the 1980s.

[1] Quote taken from: Report on regional medical programs to the President and the Congress.

Resistance in the target bacteria has swiftly followed the use of each of these agents. However, this situation has also been compounded by the indiscriminate use of antibiotics in veterinary medicine. Selection pressure exerted by these compounds has led to the rising rates of resistance in traditional hospital pathogens but has also selected for resistance in non-traditional pathogens. Unfortunately, this situation has severe repercussions in other aspects of clinical treatment. Procedures such as transplantation and surgery require antibiotic therapy and the viability of using these practices are compromised. The inevitable cost of antibiotic resistance is the loss of these valuable compounds from clinical use, leading to the dire prospect of revisiting the pre-antibiotic era.

1.2 History of Antibiotics

Until the end of the nineteenth century, traditional remedies derived from minerals and plants were used in the treatment of infectious diseases. Honey, for example, known for its anti-inflammatory and anti-microbial properties was employed in the treatment of wound infections. This natural remedy is now experiencing a resurgence in the form of granulated sugar in combination with debriding agents for the treatment of wounds (Durodie, 1984; Chirife *et al*, 1982).

Despite the obvious potential of natural potions, the search for substances with increasing antibacterial potency has always been sought. Here, Paul Ehrlich's seminal work in the first decade of this century must be mentioned. Ehrlich proposed almost all the fundamental concepts, which have governed the subsequent work on synthetic antimicrobial agents. Ehrlich recognised the need to develop synthetic

compounds that would interfere rapidly with the metabolism of infectious agents and subsequently kill these microbes but not adversely affect the host. He defined his three requirements for the treatment of infectious diseases with synthetic compounds: efficacy, convenience and low toxicity (Ehrlich, 1913). He was searching for the *therapia sterilans magna*, the one dose treatment that would cure all. His initial experiments began by using chemically synthesised agents developed by the German dye industry (Kasten, 1996). At that time three distinct lines were produced namely red, blue and yellow. The red line gave rise to the predecessors of sulphonamides (Domagk, 1935), which are used in the treatment of infectious diseases today. The second group of dyes that were produced were the aniline dyes of which methylene blue was found to demonstrate antimicrobial properties and subsequently used widely as antimalarials in the 1890s (Gutmann & Ehrlich, 1891). The yellow line of dyes gave rise to one of the most potent and widely used antimicrobial agents today: the quinolones. The initial derivatives of this yellow line were the chloroquines from which the first napthyridone nalidixic acid was synthesised. The current analogues of each of these dye lines are still used in clinical therapy today.

Ehrlich's greatest practical success came with the effective treatment for human syphilis with Salvarasan (compound 606) derived from the aniline dyes in 1910 (Ehrlich, 1910). The new drug when administered early during the course of infection was able to destroy the spirochetes. Unfortunately, Ehrlich's dream of an ideal antimicrobial agent, a 'magic bullet' was not truly realised with Salvarasan since the drug was found to be toxic in large doses with side effects, and in lower doses would cause the spirochetes to develop resistance rapidly (Kasten, 1996). Nevertheless, Salvarasan and its derivative neo-salavarasan together with bismuth

therapy were used as first line therapy against syphilis until the introduction of penicillin in the 1940s (Work & Work, 1948). In essence, Ehrlich paved the way for the use of synthetic compounds in the treatment of infectious diseases and with salvarasan proved that it could be done. The pathway to the discovery and development of sulfa drugs and penicillin clearly began with Ehrlich's pioneering research on dyes as potential chemotherapeutic agents (Ehrlich, 1913) and encouraged the work of others (Kasten, 1996) in the investigation of dyestuffs for further bactericidal and bacteriostatic properties.

As an aftermath of Ehrlich's work on dye chemotherapy, a screening programme of the stockpile of new dyes produced by the German chemical laboratories was initiated and directed by Gerhard Domagk, an experimental pathologist. In 1935, Domagk publicly announced that Prontosil, a compound consisting of a sulfonamide group attached to the dye chrysoidin, was effective in attacking streptococcal infections in animals without causing damage to the hosts (Domagk, 1935; reviewed by Otten, 1986). The enhanced antibacterial properties of sulfanilimide and the other new sulfa derivatives was a success in medicinal chemistry and further proved the therapeutic use of synthetic compounds as antibacterials (Kasten, 1996). Synthetic drug development did much for the advances in antimicrobial chemotherapy, with far reaching effects on the treatment of current infections with analogues of previously synthesised compounds.

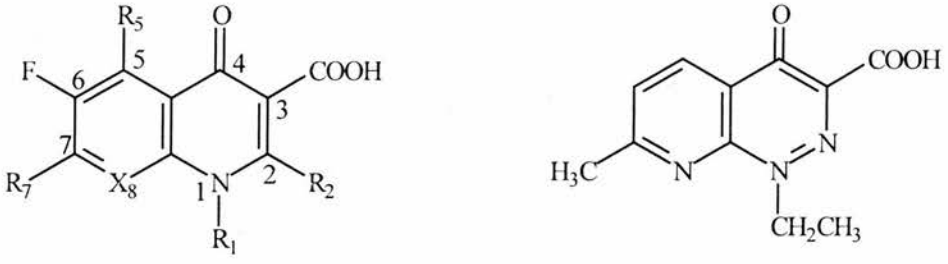
The discovery and development of the quinolone class of agents were very much in this manner. Whilst pursuing new compounds based on the structure of quinines for the treatment of malaria, a derivative of the 1,8-napthyridine molecule that possessed

antibacterial activity was produced. By 1962, George Lesher and colleagues (Lesher, 1962) had developed nalidixic acid. However, it was only in 1964 that nalidixic acid was available for the treatment of urinary tract infections and was the first quinolone of its class in clinical use (Andriole, 1991). Its poor serum tissue concentrations and its limited spectrum of activity compromised the efficacy of nalidixic acid (Norris & Mandell, 1988). Production of other quinolone compounds continued and 20 years later a significant development in the quinolone pharmacore corresponded with enhanced activity of these agents in clinical treatment. Koga *et al* (1980) observed that modifications at the C6 and C7 positions in the quinolone nucleus brought improved clinical efficacy and absorption. The subsequent addition of the fluorine atom at these positions created the more potent group of agents known as the "Fluoroquinolones". Since Lesher's initial report, 10,000 analogues of nalidixic acid and the fluoroquinolones have been synthesised. Some of these compounds have been extensively developed and used, making these agents one of the most relied upon class of antibacterials in clinical use.

1.3 Quinolones

The discovery and development of the quinolones was hailed as one of the most exciting advances in antimicrobial chemotherapy. The quinolone class of antibiotics possesses a primary structure that is known as the quinolone pharmacore (Mitscher *et al*, 1990). The quinolone pharmacore is defined as the minimum structural unit in a drug that has measurable and typical pharmacological activity (Tillotson 1996; Domagala 1994). This minimum unit itself is often of low potency and specificity. The function of the other portions of the drug molecule, like the auxopharmacophore

provides tighter receptor fit, greater selectivity, more useful pharmacokinetic characteristics and solubility of the quinolones (Mitscher *et al*, 1990). The pharmacophoric unit consists of the pyridone ring and its associated carboxyl group or surrogates as illustrated in Figure 1.1a.



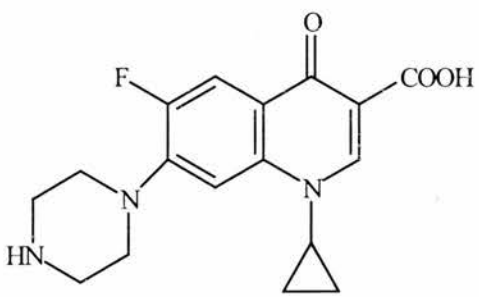
(a) Quinolone Pharmacore **(b) Nalidixic Acid**

Figure 1.1: Structure of Quinolone Pharmacore and Nalidixic Acid

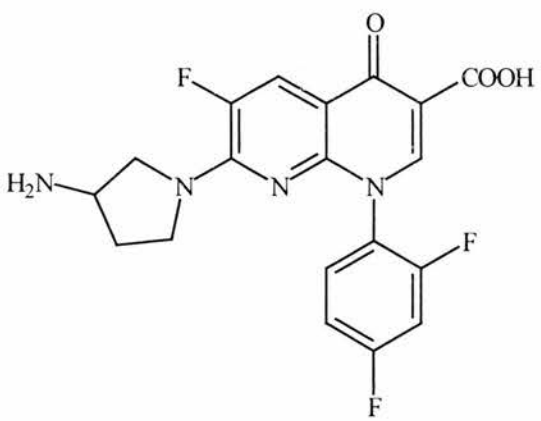
The auxopharmacore is defined by a variety of substitutions at the different carbon positions. It has been shown that modifications at certain positions such as C2, C3 and C4 could interact and markedly decrease the basic mode of quinolone drug action (Mitscher *et al*, 1990). For example, at positions 2,3,4 binding to DNA bases occurs. These bases are open to interaction with new hydrogen binding partners by the action of DNA gyrase, therefore any large chemical additions at position 2 would result in stearic hindrance at positions 3 and 4 which would compromise the action of the drug on its target (Tillotson, 1996). Therefore, very little variation is observed at the N1, C2, C3 and C4 positions. However, 3 other positions namely C6, C7 and C8 can receive a wide range of substituents that enhance the efficacy of the quinolone.

1.3.1 Position 1

The earliest quinolones possessed nitrogen-bearing modifications at position 1. This was fortuitous, as subsequent work has shown that any other substitutions like oxygen (Hogberg *et al*, 1984a) and carbon (Hogberg *et al*, 1984b) were found to be unsuitable. The substituent attached at N1 controls antibacterial potency and the cyclopropyl group in particular has been found to improve *in vivo* efficacy and activity against the anaerobes (Domagala, 1994); for example ciprofloxacin (See Figure 1.2a) has an N1- cyclopropyl substituent which gives potent activity against enterobacteriaceae and difloxacin, which possesses a N1-fluorophenyl substituent, is the most active quinolone against *Chlamydia trachomatis* (Chu & Fernandes, 1989). Against *S. pneumoniae* it has been shown that compounds that possess a 2,4-difluorophenyl at the N-1 position like tosufloxacin (See Figure 1.2b) had significantly more bactericidal activity than ciprofloxacin, which has a cyclopropyl group (Mitsuyama, 1999).



(a) Ciprofloxacin

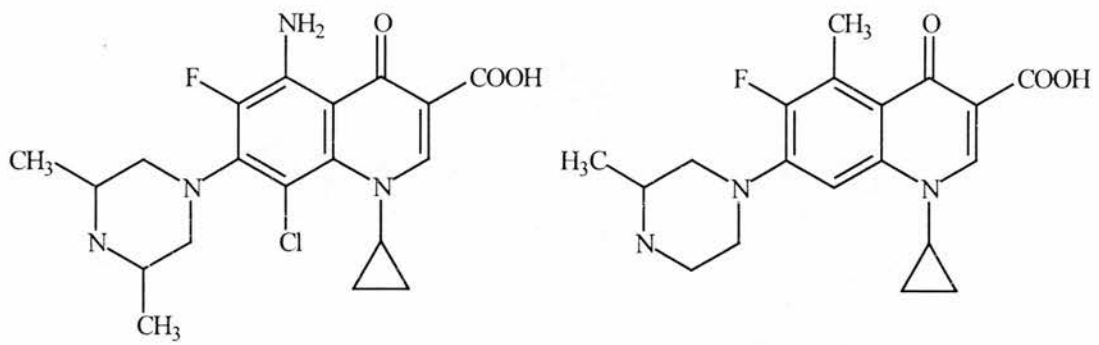


(b) Tosufloxacin

Figure 1.2: Structure of Ciprofloxacin and Tosufloxacin

1.3.2 Position 5

Initial research into the modifications at this position involved the introduction of the 1,5-napthyridines at this position. This did not yield any significant progress in the pharmacokinetics and efficacy of the quinolone (Mitscher *et al*, 1990). Recent investigations have shown that large moieties should not be introduced at this site such as fluorine, chlorine and alkyl or nitro lead to compounds with decreased activity (Jack, 1986). However, substituent combinations of (1-cyclopropyl-6-8, difluoroquinolone) as seen with sparfloxacin (See Figure 1.3a) and grepafloxacin (See Figure 1.3b) have shown increased activity against gram-positive bacteria. Both compounds also show enhanced activity against gram-positive bacteria (Domagala, 1994) with, sparfloxacin extending its potency against gram-negative bacteria.



(a) Sparfloxacin **(b) Grepafloxacin**

Figure 1.3: Structure of Sparfloxacin and Grepafloxacin

1.3.3 Position 6

It was the addition of the fluorine atom at the carbon 6 position that developed the fluoroquinolones (Koga *et al*, 1980). The introduction of the fluorine atom dramatically enhances the potency of the drug. This single atom can provide more

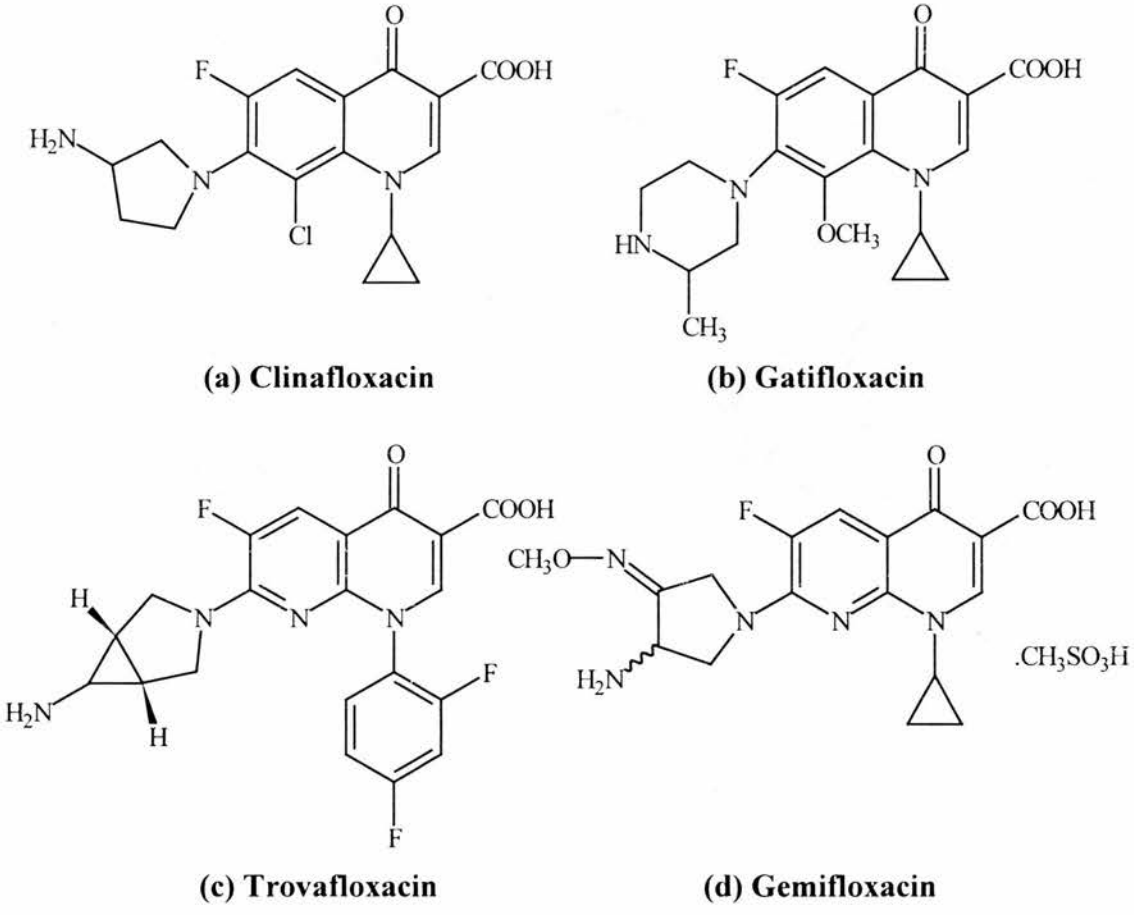
than 10 fold increases in gyrase inhibition and up to 100-fold improvement in MIC (Domagala, 1994) which is indicated by the amount of drug required to inhibit DNA gyrase and the penetration of the drug through the cell membranes in comparison to nalidixic acid.

1.3.4 Position 7

This position is the most significant within the quinolone molecule as modifications at this location can dictate the activity and pharmacokinetic profile of the drug (Domagala, 1994; Tillotson, 1996). Chemical additions at this position are linked to the quinolone nucleus via nitrogen, carbon, sulphur, or oxygen molecules (Petersen *et al*, 1996). The most common substituents at position 7 are the aminopyrolidones and the piperazines. Quinolones that have an unsubstituted piperazine ring such as ciprofloxacin and norfloxacin have been found to demonstrate good activity against gram-negatives (Sanchez *et al*, 1988), while grepafloxacin, which has a 3-methyl piperazine and sparfloxacin with a 3,5-dimethyl piperazine (Perry *et al*, 1999), contain substituted piperazine rings which exhibit greater activity against gram-positives and are believed to provide increased penetration into bacterial cells (Domagala, 1994).

Generally, aminopyrolidine modifications confer increased protection against gram-positive bacteria but a pyrolidine ring without the amino group is detrimental to activity (Domagala, 1994). Generally, compounds that possess the 7-amino-pyrolidine ring are more active against gram-positives than those that possess a 7-piperazine ring. Clinafloxacin (See Figure 1.4a) which has a 3-amino pyrolidine substituent (Mitsuyama, 1999) and gatifloxacin (See Figure 1.4b) which has a 3-

amino, 4-methyl substituent (Appelbaum & Hunter, 2000). Both trovafloxacin (See Figure 1.4c) (Brighty & Gootz, 1997) and clinafloxacin (Ednie *et al*, 1999) have enhanced activity over ciprofloxacin against gram-positive cocci and anaerobes whilst maintaining good activity against gram-negative pathogens (Chu & Fernandes, 1989). Gemifloxacin (See Figure 1.4d) possesses an unusual pyrolidine ring that has a 3-aminomethyl and 4-methyloximino substituent which has been linked to increased antibacterial activity against gram-positive bacteria (Hong *et al*, 1998).



(a) Clinafloxacin **(b) Gatifloxacin**

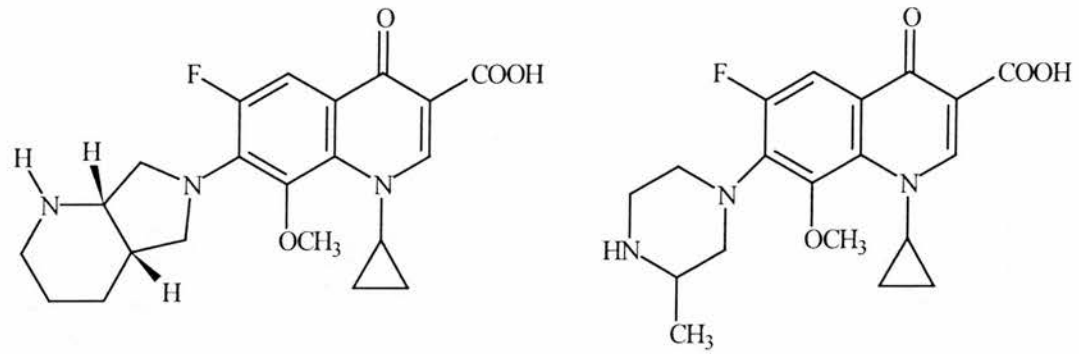
(c) Trovafloxacin **(d) Gemifloxacin**

Figure 1.4: Structure of Clinafloxacin, Gatifloxacin, Trovafloxacin, and Gemifloxacin

The other unusual side chain substituent at position 7 is a bicyclic ring fused to the pyrolidone ring, which is found in both trovafloxacin and moxifloxacin (Child *et al*, 1995; Dalhoff, 1999; Appelbaum & Hunter, 2000). Both these compounds demonstrate good activity against gram-positives (Dalhoff, 1999; Gootz *et al*, 1996).

1.3.5 Position 8

Substitutions at this position has been implicated in marked improvements in activity against both gram-positive and anaerobic bacteria (Domagala, 1994), although activity against gram-negative bacteria is reduced. The most productive substituents with respect to pharmacokinetics and potency are the fluoro, chloro and methoxy groups as other groups tend to decrease both *in vitro* and *in vivo* efficacy (Tillotson, 1996; Domagala, 1994). In addition, to the enhanced antibacterial activity, these compounds have also been found to interact more specifically with humans. The substitutions at this site have been associated with the severe side effects of phototoxicity that fluoroquinolone use is able to cause (Paton & Reeves, 1991). The naphthyridones have a nitrogen in the place of the carbon in the ring as seen in trovafloxacin and gemifloxacin. Quinolones that possess changes at the C8 position are ciprofloxacin, clinafloxacin, moxifloxacin (See Figure 1.5a), and gatifloxacin (See Figure 1.5b) respectively. Gatifloxacin and moxifloxacin both possess the methoxy substitution at the C8 position which confers good anaerobic activity and studies indicate negligible phototoxic potential (Perry *et al*, 1999; Dalhoff, 1999).



(a) Moxifloxacin **(b) Gatifloxacin**

Figure 1.5: Structure of Moxifloxacin and Gatifloxacin

1.3.6 Quinolone Generations

The successful and widespread use of ciprofloxacin has demonstrated the potential of the fluoroquinolones in the treatment of a wide range of infections although activity against gram-positive infections has been variable. Thus, the major thrust of fluoroquinolone research has been to develop compounds with improved activity against gram-positives such as staphylococci, particularly MRSA and streptococci. Each quinolone generation has been designed to maintain and enhance activity whilst minimising the risk of adverse effects. The quinolone progenitors namely the napthyridone and the quinolone pharmacore (See Figure 1.1a) are structurally similar and subsequent agents have been developed on the basis of these fundamental structures.

1.3.6.1 Generation I

The first generation quinolone, flumequine possessed anti-pseudomonal activity and was used until reports of ocular toxicity removed it from clinical use (Rohlfing *et al*, 1976). The first generation napthyridone derivative nalidixic acid was used widely in

the treatment of urinary tract infections caused by gram-negative bacteria but was subsequently removed because of poor serum and tissue concentrations (Andriole, 1991). Resistance to both agents developed rapidly, thereby rendering them useless against bacterial pathogens.

1.3.6.2 Generation IIa

The next fluoroquinolone derivative, ciprofloxacin had predominantly gram-negative activity and has been widely available since the 1980s (Thomson, 1999). The addition of the piperazine group at position 7 was instrumental in the enhanced potency of this antibiotic. The launch and use of ciprofloxacin revolutionised the treatment of gram-negative infections like bacteriaemia (Krumpe *et al*, 1999). Ciprofloxacin also possessed exceptional activity against pseudomonal infections in children with cystic fibrosis (Campoli-Richards *et al*, 1988). The only shortfall of ciprofloxacin was its reduced and variable activity against gram-positive bacteria especially *S. pneumoniae* and methicillin resistant *Staphylococcus aureus* (MRSA) (Ball, 1994). Certain adverse effects were associated with ciprofloxacin use such as CNS effects and photoxicity (Domagala, 1994).

1.3.6.3 Generation IIb

This generation of quinolones was developed with activity against both gram-positive and gram-negative bacteria, thereby enabling this class of agents to be used against the pneumococcus. Grepafloxacin is an example of a IIb quinolone, which possesses activity against both gram-negative and gram-positive pathogens. Grepafloxacin differs from ciprofloxacin by the 2-methyl group substitutions it

possesses at position 5 at the heterocyclic ring and in the piperazine ring at C7. The methyl substituent at position 7 has been associated with enhanced gram-positive activity as well as increased phototoxic potential (Stahlman & Schwabe, 1997). This property has underlined the removal of sparfloxacin/grepafloxacin from clinical use. The napthyridone derivatives enoxacin and tosufloxacin were also removed from clinical treatment after reports of increased resistance and severe adverse effects (Hooper, 1998).

1.3.6.4 Generation IIIa

Examples of generation IIIa are compounds like gatifloxacin and moxifloxacin. Structurally, gatifloxacin and moxifloxacin are similar although moxifloxacin possesses an azabicyclo substitution at position 7, in contrast to gatifloxacin, which retains a piperazinyl group. Both these agents have excellent activity against *S. pneumoniae* regardless of penicillin and macrolide sensitivities (Bruggemann *et al*, 1997) and have no reported adverse effects (Balfour & Wiseman, 1999; Perry *et al*, 1999). In contrast, agents such as trovafloxacin (napthyridone derivative) and clinafloxacin both of which share structural similarities have been shown to induce severe hepatotoxicity and hypoglycaemia in humans. Both agents have been subsequently withdrawn from clinical therapy (Ball, 2000).

1.3.6.5 Generation IIIb

Gemifloxacin which is a napthyridone derivative with a 1-cyclopropyl group is the only drug of this generation. Gemifloxacin has markedly better activity against both

gram-positive and negative bacteria and in some cases has been found to surpass the action of IIIa quinolones in *in-vitro* studies (Marchese *et al*, 2000).

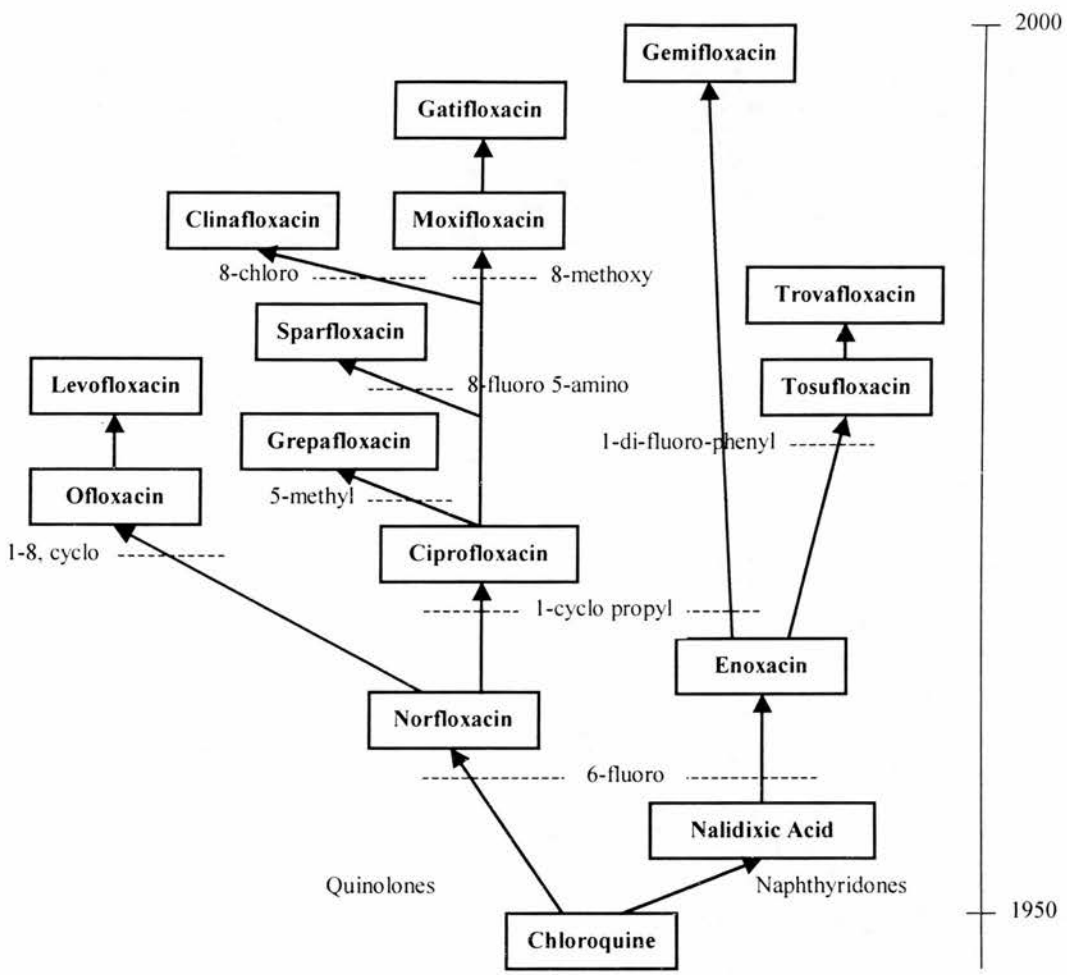


Figure 1.6: Diagrammatic Representation of the Quinolone Generations
(Adapted from Wise, 2000)

Figure 1.6 shows the evolution of quinolone compounds beginning with agents with modest activity against gram-negatives to broad-spectrum activity against both gram-positive and negative bacteria.

1.3.7 Structure and Toxicity Relationships

Therapeutic drug use has always been associated with some form of adverse reaction in man. One of the initial side-effects implicated with quinolone use was arthropathic damage in weight-bearing joints of animals when high doses of the drug were administered over a prolonged period (Ingham *et al*, 1977). The most severe adverse reaction that has caused fluoroquinolones to be removed from clinical use is phototoxicity. Phototoxicity has been found to be almost always non-immunogenic (Paton & Reeves, 1991) and appears to be a class effect for most of the known quinolone agents although they differ markedly from each other in the levels of phototoxic risk inducible in humans (Domagala, 1994). Quinolone photoreactivity is oxygen dependent. Generally, when quinolones are exposed to UVA radiation, three molecular outcomes may result: the quinolone gradually decomposes (Matsumoto *et al*, 1992), the quinolones passes light energy onto oxygen forming singlet oxygen, which is a powerful oxidizing agent and radical generator (Robertson *et al*, 1991), or the quinolone returns to its ground state unchanged (Foote, 1976). The quinolones, which are the most phototoxic are those that induce singlet oxygen and radicals subsequently causing severe tissue damage (Foote, 1976).

Quinolones, which reach high concentrations or accumulate in the skin, have a higher risk of producing phototoxic events. It has been shown that the possession of a halogen, such as fluorine or chlorine at position 8 of the fluoroquinolone nucleus contributes to severe phototoxicity (Domagala, 1994). Understandably, the phototoxic potential of a drug has a direct impact on its therapeutic properties. Compounds that have shown exceptional *in vitro* antibacterial activity but phototoxic potential have been dropped from further development as seen with BAY Y3118 or

with the recommended limited clinical use of sitafloxacin and clinafloxacin. Newer compounds such as moxifloxacin and gemifloxacin which exhibit similar *in vitro* activity as the afore-mentioned compounds but alternate C8 substitutions are still capable of inducing a mild phototoxic reaction which is within the safety margins associated with quinolone use (Man *et al*, 1999).

The previous decade has seen the removal of several fluoroquinolones from clinical use after being awarded the 'pink letter', which warned all doctors of the phototoxic and carcinogenic potential of the drugs. Sparfloxacin was removed from clinical treatment in October 1994 due to reports of phototoxicity and photocarcinogenicity (Ministry of Health & Welfare Bulletin, Japan, 1994). More recently, grepafloxacin was found to induce severe cardiovascular episodes in patients prior to its removal from clinical treatment (Glaxo Wellcome, 1999). Fourteen cases of severe liver failure were reported and implicated with trovafloxacin use. Although trovafloxacin has not been removed from clinical therapy, caution is advised when administering to patients (Nightingale,1999). Quinolone use is able to induce other side effects that include gastrointestinal upset, skin rash and arthropathy. These side effects are mediated by this class of agents and cannot be moderated by molecular variation (Tillotson 1996; Domagala 1994).

1.3.8 Drug-drug Interactions with Fluoroquinolones

Quinolones are able to interact with theophylline or non-steroidal anti-inflammatory drugs (NSAIDS). Quinolones with alkylated piperazines and pyrolidines such as clinafloxacin and trovafloxacin have fewer effects with NSAIDs than those with unsubstituted piperazines like ciprofloxacin (Tillotson, 1996). Since the piperazine

ring at position 7 mimics the structure of γ-aminobutyric acid (GABA) it is able to antagonise GABA receptor binding in the brain, thereby resulting in heightened CNS excitation (Tillotson, 1996). Despite the inhibitory activities of fluoroquinolones on GABA neurons, there are few reported NSAID-induced CNS effects associated with quinolone use.

Enoxacin was the first quinolone reported to increase theophylline concentrations leading to severe adverse reaction (Wijnands *et al*, 1984). Quinolone and theophylline interaction occurs, when the metabolic breakdown pathway mediated by cytochrome p450 enzymes is inhibited by quinolones thereby causing an accumulation of theophylline which subsequently results in convulsions. These side effects have been observed most frequently with quinolone compounds that possess substituents at position 7 (Tillotson, 1996).

1.3.9 Anti-gram Positive Quinolones

The majority of fluoroquinolones that have been in clinical use till recently, possessed excellent *in vitro* and *in vivo* activity against gram-negative organisms and moderate activity against gram-positive bacteria. These older agents have been used in the treatment of selected respiratory tract infections, but concerns have remained regarding their efficacy in infections caused by marginally susceptible organisms like *Streptococcus pneumoniae* (Legg & Bint, 1999). Ciprofloxacin, the forerunner of the fluoroquinolones has been used with variable success against pneumococcal pneumonia. Reports of therapeutic failure with ciprofloxacin has led clinicians to question the efficacy of quinolones as first-line empirical therapy in the treatment of respiratory tract infections caused by the pneumococcus and other gram-positive

pathogens (Perez-Trallero *et al*, 1990; Kuehnert *et al*, 1999; Barry *et al*, 1999). This scepticism was further validated by the microbiological eradication success rate being more pronounced with ß-lactams such as amoxycillin and penicillin rather than ciprofloxacin for the pneumococcus (Balfour & Wiseman, 1999). The limitations of quinolones such as ciprofloxacin coupled with increasing penicillin and macrolide resistance in *S. pneumoniae* (Felmingham & Washington, 1999) urgently indicates the need for new treatment options. This has prompted pharmaceutical companies to focus on the development of broader spectrum fluoroquinolone agents with enhanced activity against gram-positive pathogens. In the last two years, antimicrobial agents such as moxifloxacin and gatifloxacin with enhanced activity against gram-positive bacteria have been launched. This is timely, as respiratory tract infections (RTI) are prime indications for antibacterial prescribing both in the community and hospital and where current recommendations for the treatment of RTIs include the single use of a drug and combination regimens (Finch, 1995). The new fluoroquinolones are principal candidates for this, as they possess a broad antibacterial spectrum and rapid bactericidal activity.

1.3.10 Moxifloxacin

Moxifloxacin is an 8-methoxy quinolone which is an enantiometrically pure 6-fluoro, 8 methoxy quinolone carboxylic acid (Balfour & Wiseman, 1999) (See Figure 1.5a). Due to its C7 and C8 modifications it possesses enhanced activity towards gram-positive pathogens, particularly, the pneumococcus (Balfour & Wiseman, 1999). *In vitro* testing has shown that moxifloxacin demonstrates good activity against *S. pneumoniae* regardless of penicillin and macrolide susceptibilities and was found

to be 8-fold more active compared to both ciprofloxacin or levofloxacin (Balfour & Wiseman, 1999; Dalhoff, 1999). It is also as potent as ciprofloxacin against other gram-negative respiratory pathogens such as *H. influenzae* and *M. catarrhalis* (Dalhoff *et al*, 1996). The currently reported MIC_{50} and MIC_{90} values of *S. pneumoniae* against moxifloxacin are 0.12-0.25mg/l (Dalhoff, 1999) which is well below the recommended breakpoint value for moxifloxacin (Andrews *et al*, 1999). The clinical success of the new fluoroquinolone agents is dependent on their efficacy against pathogens previously resistant to older agents of the same class. Moxifloxacin was able to retain its potency against isolates with reduced susceptibility to ciprofloxacin with an MIC_{50} of 0.25mg/l (Piddock *et al*, 1998; Pong *et al*, 1999). The correlation of *in vitro* with *in vivo* activity is crucial in determining the clinical utility of any antibiotic. *In vivo* studies have shown that moxifloxacin administered once a day achieved bacteriological success rates of approximately 90% or higher in community acquired pneumoniae (CAP) and acute exacerbation of chronic bronchitis (AECB) (Balfour & Wiseman, 1999). This single dosing regimen of 400mg of moxifloxacin was as effective as 1g of amoxycillin three times a day (Balfour & Wiseman, 1999). In comparison with cefuroxime axetil, moxifloxacin had a 11% higher success rate in the eradication of *Streptococcus pneumoniae* (Siegert *et al*, 2000). Post antibiotic effect of more than 1hr was observed at 4× MIC against *S. pneumoniae, E. coli,* and *H. influenzae* with moxifloxacin (Balfour & Wiseman, 1999). This improved activity observed is attributed to the bulky C7 substituent of moxifloxacin, which in other quinolones like sparfloxacin and ciprofloxacin has also been linked to enhanced efficacy (Chu & Fernandes, 1989; Balfour & Wiseman, 1999; Hunter & Appelbaum, 2000). The C8 methoxy group

confers light stability therefore moxifloxacin has a low propensity for causing phototoxic or CNS excitatory effect (Balfour & Wiseman, 1999). The only side effect implicated with moxifloxacin use is gastrointestinal disturbance (von Keutz & Schulter, 1999).

1.4 Bacterial Topoisomerases

DNA topology modulates every physiological function within the cell. In bacteria, this topology determines the essential cellular processes such as DNA replication and transcription levels of various promoters. These cellular functions are dependent on the appropriate level of DNA supercoiling being maintained in the cell (Wang, 1985). In bacteria, these negative superhelical turns are introduced into DNA by a class of enzymes known as DNA topoisomerases.

Topoisomerases fall into two general categories: Type I and Type II. This categorisation is based on the biochemical mechanisms responsible for DNA strand passage. Type I topoisomerases are monomeric enzymes which include topoisomerase I and III. In *E. coli*, topoisomerase I and III are active as dimers consisting of a single type subunit encoded by *topA* and *topB* genes (Gellert, 1981; Hooper, 1998). Topoisomerase I and III have the ability to unlink or decatenate interlocked DNA circles and do so by characteristically catalysing single stranded DNA cleavage and strand passage in the absence of ATP (i.e. removing negative superhelical twists without ATP) (Hooper, 1998; Roca 1995).

Type II topoisomerases encompass DNA gyrase and topoisomerase IV in bacteria and topoisomerase II in eukaryotic cells. These enzymes are multi-subunit proteins,

which require energy cofactors for activity (Hooper, 1998; Roca 1995). Unlike type I topoisomerases, DNA gyrase and topoisomerase IV mediate double-strand breakage with the passage of another helix through the transient break by a process requiring ATP hydrolysis (See Fig.7) (Drlica & Zhao, 1997; Watt & Hickson 1994). These reactions are essential in maintaining the appropriate state of DNA supercoiling and separation of daughter DNA molecules during cell division. Type I and type II topoisomerases are found in all bacterial cells and act opposingly on superhelical DNA, to maintain the optimal DNA conformation within the cell (Gellert, 1981). Major interest in these enzymes particularly the type II topoisomerases stem from the fact that they play an essential role in DNA maintenance and are primary cellular targets for some of the most widely prescribed antibiotic and anticancer agents currently used in the treatment of human disease.

1.4.1 DNA Gyrase

1.4.1.1 GyrA Subunit

Gellert *et al* (1976) first isolated the Type II DNA topoisomerase, DNA gyrase from *E. coli*. DNA gyrase is a multi-subunit enzyme consisting of two GyrA and GyrB subunits, encoded by *gyrA* and *gyrB* genes respectively where the active enzyme presents as an A_2B_2 complex. All enzyme activities requires both subunits but certain domains mediate both functions (Gellert, 1981). Mechanistically, gyrase binds to DNA, where a segment of approximately 130bp wraps around the protein. This wrapped DNA is cleaved in both strands with a 4-base pair stagger between the break sites, which results in the formation of DNA-protein covalent bonds between the GyrA subunits and the DNA (Roca, 1995) (See Figure 1.7). Another segment of

DNA is passed through this double-stranded break before it is resealed (Mizuuchi *et al*, 1980). Catalytic supercoiling mediated by DNA gyrase A requires the hydrolysis of ATP (See Figure 1.8). The fact that DNA gyrase breaks both strands of DNA is unique in comparison to the other type 1 topoisomerases that nick only one strand. A further distinction is that the cleavage that DNA gyrase institutes is highly site specific (Fisher *et al*, 1981; Morrison *et al*, 1979).

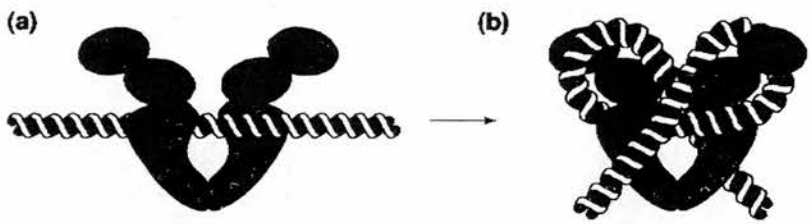


Figure 1.7: Mechanistic Action of DNA Gyrase
(Taken from Roca, 1995)

(a) DNA gyrase binds to the DNA. Unlike other Type II topoisomerases DNA gyrase wraps around the flanking DNA regions, thereby bringing the amino terminal ends of the enzyme to the entrance of the T-segment of DNA.
(b) Upon ATP binding, the DNA flanks are captured and transported. The inversion of the positive node facilitates negative supercoiling of DNA.

In *E. coli,* DNA gyrase A mediated strand breakage and reunion has been found to occur at Tyrosine-122 (Horowitz & Wang, 1987). *In vivo* studies have shown that DNA gyrase is essential for DNA replication and is involved in both initiation and elongation (growing–point propagation) of DNA as determined by the patterns of replication arrest observed in both *gyrA* and *gyrB* conditional lethal mutants (Wang, 1985).

In DNA replication, gyrase functions by introducing negative supercoils, to facilitate binding of initiation proteins to DNA and enhancing DNA strand unwinding during fork propagation. Inactivated DNA gyrase in dividing bacteria accumulates partially

segregated nucleoids that completely segregate upon addition of purified DNA gyrase (Wang, 1985; Fisher *et al*, 1981).

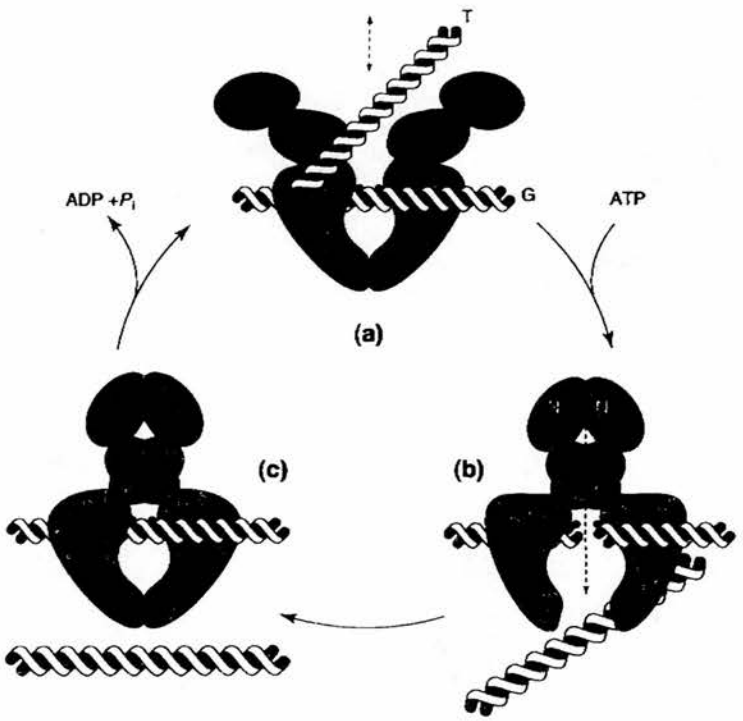


Figure 1.8: Mechanistic Action of Type II DNA Topoisomerases
(Taken from Roca, 1995)

Two gate mechanism of type II topoisomerases.
(a) DNA gyrase forms an open clamp, which binds to the DNA duplex enabling the second duplex DNA to lodge in the G-segment, bound enzyme leaving the clamp open.
(b) The binding of ATP shuts the protein gates, which are composed of the amino-terminal domains of each homodimer.
(c) With the T segment outside, the second gate closes and the rejoined G-segments remains inside the protein. The enzyme returns to the open clamp form after ATP hydrolysis and release of products.
N: denotes the amino terminal domains of the enzyme
G & T segments: indicates independent segments of DNA.

DNA gyrase is also involved in transcription at least indirectly, because negative supercoiling of DNA can increase expression of some operons and decrease expression of others. In addition, the enzyme may also be involved more directly in the removal of positive supercoils accumulating ahead of RNA polymerase as it progresses along DNA templates (Wang, 1985). In short, damage in DNA gyrase

results in the complete inhibition of replicative DNA synthesis and subsequently results in cell death (Cozzarelli, 1980).

1.4.1.2 GyrB Subunit

GyrB, is a 90-kDa fragment in *E. coli* which complexes with GyrA and functions by supporting DNA relaxation but not supercoiling or ATP hydrolysis (Wang, 1985). The N-terminal domain of GyrB contains the ATP binding site as elucidated with X-ray crystallography and also has been found to be homologous to the amino terminal part of the eukaryotic topoisomerase II (Drlica & Zhao, 1997; Hooper, 1998). The ATPase activity of DNA gyrase B is competitively inhibited by the coumarin derivatives such as novobiocin which are structurally unrelated to quinolones (Maxwell, 1993). It has been shown that genetic mutations associated with novobiocin resistance are clustered around the 5' (N-terminal) region of the GyrB gene as described in *S. pneumoniae* (Munoz *et al*, 1995). However, genetic mutations sustained within the C-terminal region of the subunit are associated with quinolone resistance as seen in *E. coli* (Yoshida *et al*, 1991) and *S. pneumoniae* (Pan & Fisher, 1998). It appears that apart from the ATPase activity, GyrB is also involved in DNA binding and strand passing (Cozzarelli, 1980).

In combination, the A and B subunits of DNA gyrase are able to introduce supercoils by relaxing negative supercoils in the presence of ATP and positive supercoils in the absence of ATP in addition to the catenation and decatenation of DNA within a bacterial cell.

1.4.2 Topoisomerase IV

In 1990, Kato *et al* discovered a homolog of gyrase that was subsequently called topoisomerase IV. Topoisomerase IV bears a similar arrangement to DNA gyrase. It consists of 2 ParC and 2 ParE subunits (Kato *et al*, 1990). Analysis of the *E. coli* nucleotide sequences of *parC* and *parE* genes indicates homology to the A and B subunits of DNA gyrase respectively and that the *parE* gene is located upstream of *parC* (Peng & Marians, 1993). Homology between DNA gyrase and topoisomerase IV is also seen in gram-positive bacteria like *S. pneumoniae* (Pan & Fisher, 1996b).

Like other topoisomerase II enzymes, topoisomerase IV also employs a double strand mode of passage (Roca, 1995). However, the fundamental means by which this is achieved differs between DNA gyrase and topoisomerase IV. DNA gyrase wraps itself around the DNA unlike topoisomerase IV (Peng & Marians, 1995). This mode of wrapping favours intra-molecular bonding rather than intermolecular bonds which has been found to have a deleterious impact on the decatenating activity of the enzyme (Peng & Marians, 1995) as is the case with DNA gyrase A where the decatenating potential of this enzyme is poor in comparison to its supercoiling and relaxing activities.

The superior DNA decatenating capacity of topoisomerase IV relative to gyrase could explain the functional differences between the enzymes. The primary feature of topoisomerase IV is its ability to remove catenanes before the round of DNA replication is complete in comparison to DNA gyrase, where this is only done after the replication cycle is complete (Hooper, 1998). Studies with *E. coli* and

S. typhimurium that sustain temperature sensitive mutations in topoisomerase IV, were found to accumulate supercoiled catenated plasmids *in vivo* within minutes of the non-permissible temperature (Kato *et al*, 1990). Furthermore, mutations within *parC* and *parE* subunits resulted in mutants with cellular phenotypes consistent with the absence of an enzyme involved in chromosomal segregation (Adams *et al*, 1992).

Electron microscopy has shown that although DNA gyrase is able to supercoil catenanes, which are products of DNA synthesis in cells, it is unable to unlink them. It is now apparent that catenanes borne out of the processes of recombination and tangling are unlinked by DNA gyrase and those arising from replication are resolved by topoisomerase IV (Adams *et al*, 1992). Deficiencies in topoisomerase IV lead to an accumulation of catenated plasmids in both *S. typhimurium* and *E. coli* cells which suggests a stage in chromosomal partitioning catalysed by topoisomerase IV for which DNA gyrase cannot substitute (Kato *et al*, 1990). It has also been shown that DNA synthesis is not significantly affected in topoisomerase IV mutant *S. typhimurium* cells although they sustain severe defects in chromosomal partitioning. In contrast, inactivation of DNA gyrase introduced by mutation or drug binding has been shown to severely inhibit DNA synthesis resulting in cell death (Adams *et al*, 1992; Khodursky *et al*, 1995). This is validated in nature where bacterial species like *Mycobacteria* spp and *Helicobacter* spp (Hooper, 1998) do not possess topoisomerase IV, which implies that either DNA gyrase or an equivalent of topoisomerase IV have compensated for the absence of the enzyme. These observations indicate a division of labour between the type II bacterial topoisomerases when both are present and given the level of homology shared

between the enzymes it is possible that there may be an overlap in function where only one is present.

1.4.3 Intracellular Drug Targets

Despite differences in both biochemical and catalytic characteristics, the crucial feature common to all topoisomerases is the strand passage event. The ability to pass a single or double stranded DNA segment freely through another comes with a heavy price. To maintain genomic integrity during this cleavage event, topoisomerases attach covalently via phosphotylrosyl bonds to the newly synthesised DNA. Usually, these covalent-DNA enzyme complexes are temporary catalytic intermediates, which exist in low intracellular concentrations within the cell (Maxwell, 1997; Froelich-Ammon & Osheroff, 1995) and are subsequently, tolerated by the cell. There are certain physiological conditions and natural or synthesised compounds that are able to prolong the lifespan of these catalytic intermediates resulting in severe damage to the cell. This unique and essential function of topoisomerases within bacterial cells secures the role of these essential enzymes as excellent drug targets (Drlica & Franco, 1988) and has been exploited by pharmaceuticals resulting in the production of potent classes of antibiotics and anti-cancer agents (See Table 1.1).

Class of Drug	Examples	Topoisomerases Inhibited
Coumarins	Novobiocin, coumermycin, chlorobiocin	Bacterial Gyrase (B subunit) Eukaryotic topoisomerase
Quinolones	Nalidixic Acid, Oxolinic Acid, Norfloxacin, Ciprofloxacin	Bacterial Gyrase (A subunit) Topoisomerase IV (Subunit C/ E)
Acridines	N-AMSA	Eukaryotic topoisomerase II
Alkaloids	Camptothecin	Eukaryotic topoisomerase II

Table 1.1: Bacterial and Eukaryotic Topoisomerase II Inhibitors
(Adapted from Drlica & Franco, 1988)

Topoisomerase II inhibitors can be divided into either topoisomerase poisons or inhibitors. Quinolones are termed "topoisomerase poisons" as they disrupt the DNA-breakage reunion reaction, thus disrupting DNA supercoiling. Kreuzer and Cozzarelli (1979) proposed the " poison hypothesis" where the interaction of the quinolone with DNA gyrase converts it into cellular poison. This cellular poison is presumed to be the quinolone-DNA-gyrase complex, which inhibits the passage of RNA polymerases, thereby inhibiting DNA transcription (Kreuzer & Cozzarelli, 1979; Willmott *et al*, 1994). Coumarins, on the other hand, interact with the ATPase subunit of DNA gyrase without trapping the DNA enzyme reaction intermediate and are termed "topoisomerase inhibitors"(Gellert, 1976; Drlica & Franco, 1988). The eukaryotic topoisomerase inhibitors are purported to work in a similar manner to the quinolones (Hsiang *et al*, 1985).

1.5 Quinolone Mode of Action

1.5.1 Mode of Binding

Quinolone resistance mutations have been shown to occur in the genes encoding both DNA gyrase and topoisomerase IV clearly implying a role for both enzymes in quinolone binding. However, drug binding studies have been unable to pinpoint the exact position of binding within these enzymes to the quinolone. Most investigations have sought to elucidate the interactions of DNA gyrase with the quinolones, although relatively little is known about the mechanistic aspects of interaction with topoisomerase IV. Given the level of homology between both the enzymes, it may be possible to speculate that the quinolone enzyme interactions would be similar.

1.5.2 Binding of Quinolones to DNA

The initial experiments aimed at elucidating the mode of quinolone action measured the binding of radio-labelled norfloxacin to calf thymus DNA using the equilibrium dialysis method (Bourguigon *et al*, 1973). No binding was detected in these experiments indicating that nalidixic acid did not bind to double stranded or supercoiled DNA. Shen & Pernet (1985) repeated this experiment with radiolabelled norfloxacin and found that both equilibrium dialysis and membrane filtration techniques failed to detect any appreciable level of drug binding to DNA gyrase. Further analysis showed that quinolones bound poorly to relaxed double stranded DNA instead of supercoiled DNA (Palu *et al*, 1988). Characterisation of this interaction has shown that the binding of norfloxacin to DNA preferentially occurred in the presence of single stranded DNA. This observation indicated that quinolone-

DNA interaction occurred via hydrogen bonding to the exposed bases in the single stranded region (Shen *et al*, 1989a) thereby suggesting that drug binding to relaxed double stranded DNA was weak in contrast to drug binding to supercoiled DNA. The drug binding to supercoiled DNA was subsequently described to occur in a highly co-operative manner to a saturable site (Shen *et al*, 1989a). Subsequent experiments have confirmed this quinolone-DNA interaction, albeit in a magnesium dependent manner (Palu *et al*, 1992).

1.5.3 Effect of DNA Gyrase on Quinolone Binding

Several studies have now confirmed that quinolones do not show significant levels of binding to either DNA gyrase or DNA alone (Shen & Pernet, 1985; Palu *et al*, 1992; Critchlow & Maxwell, 1996) but do so to the DNA gyrase-DNA complex. The conditions in which binding occurs are selective, requiring the presence of a non-hydrolysable ATP analogue ADPNP to a mixture of DNA gyrase and DNA (Shen *et al*, 1989b). Similarities between quinolone binding to supercoiled DNA via a saturable site and the formation of a saturable quinolone binding site after the binding of DNA gyrase and variable DNA forms (i.e. linear/relaxed) led to the proposal of the following model (Shen *et al*, 1989c).

1.5.4 Co-operative Quinolone-DNA Binding Model.

Shen *et al* (1989c) proposed this model based on the interaction of the quinolone DNA and gyrase. The binding of DNA gyrase to relaxed DNA cleaves both strands with a 4bp stagger in the presence of ATP. The quinolones bind to the Tyr122 region, the active site of DNA gyrase, via hydrogen bonding between the single

stranded DNA and the carbonyl and carboxyl groups at positions 3 and 4 on the quinolone nucleus (Shen *et al*, 1989c) (See Figure 1.9). Four or more drug molecules are thought to bind to the DNA in the pocket by forming strong intermolecular bonds (See Figure 1.9a).

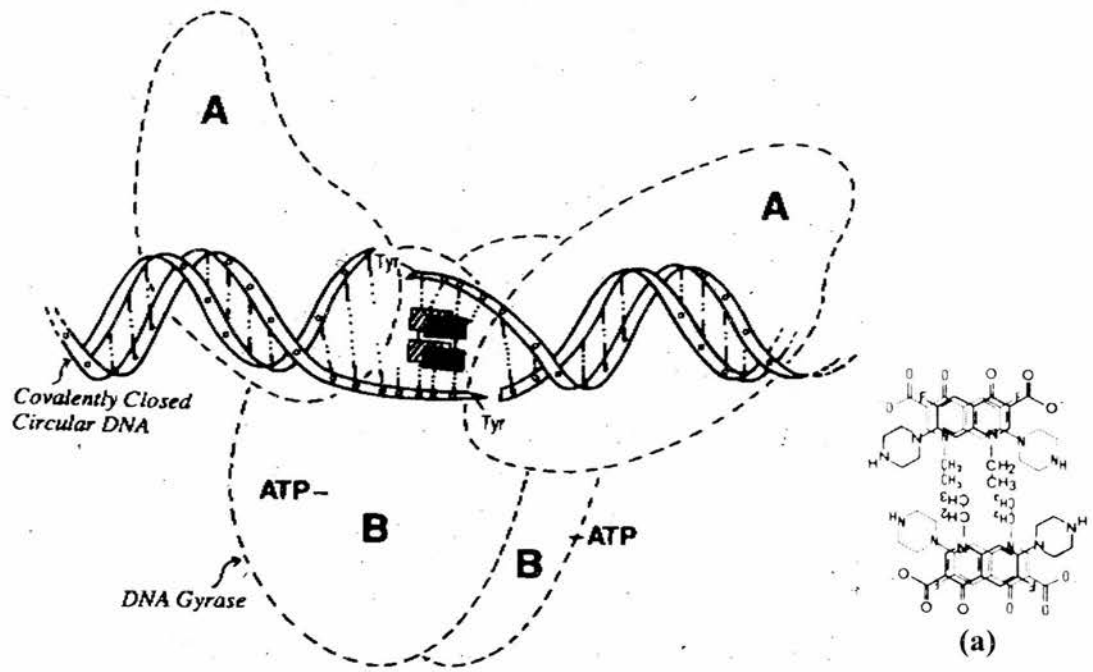


Figure 1.9: Cooperative Quinolone DNA Binding Model
(Taken from Shen *et al* (1989c))

Co-operative quinolone-DNA binding model proposed by Shen *et al* (1989c). Filled and hatched boxes represent the quinolone molecules inside a gyrase-induced single-stranded DNA binding site. Figure 1.9a represents the quinolone molecule stacking in the co-operative quinolone-DNA binding model.

The drug binding occurs either by ring stacking of quinolone rings which are hydrogen bonded to the same DNA strand or by tail to tail hydrophobic interactions with the N-1, C-2 and C-8 substituents of the quinolone molecules which are hydrogen bonded to single stranded DNA (Shen *et al*, 1989c). Shen *et al* (1989c) postulated that substituents within the quinolone structure, particularly at the C-7 position were involved in the drug-enzyme interactions. This model hinges on DNA

cleavage by DNA gyrase. Studies involving DNA gyrase mutants with substitutions at tyrosine 122, were found to bind quinolones at similar levels to the wild type DNA gyrase (Reece & Maxwell, 1991; Critchlow & Maxwell, 1996). From these results, it is clear that DNA cleavage may not be a necessity in the formation of the DNA-gyrase and quinolone complex thereby contesting the model proposed by Shen *et al* (1989c).

1.5.5 Magnesium Bridge Model

The requirement of a fixed concentration of magnesium ions for norfloxacin binding to DNA has been demonstrated by Palu *et al* (1992). The model proposes that quinolone binding to DNA occurs via a magnesium bridge rather than hydrogen bonds (See Figure 1.10). The quinolones are stabilised through the stacking interactions between the single stranded DNA base and the quinolone planar system. Unlike the Shen model, no cooperativity is required for the drug-DNA-gyrase complex formation, although both models concur that quinolone interactions occurs via either the C-7 and N-1 substituents of the quinolone nucleus. Further experiments have confirmed the presence of magnesium ions between the carboxylate and ketone groups of the quinolone molecule (Lecomte *et al*, 1994) and phase partitioning has shown that the binding of DNA to fluoroquinolones is dependent on the drug's ability to bind magnesium ions (Bazile-Pham Khac & Moreau, 1994).

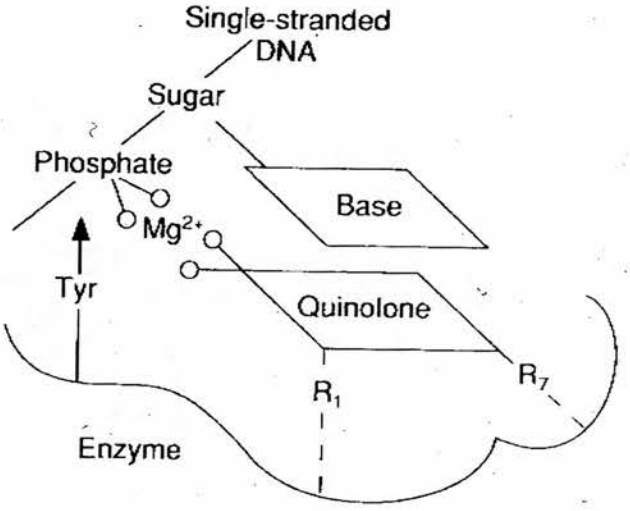


Figure 1.10: Schematic Representation of the Palumbo Model.
(Taken from Palumbo *et al*, 1993)

Quinolone drugs are proposed to bind via a magnesium ion bridge between the C-3 and C-4 groups and the phosphate groups of DNA. Interactions between the gyrase and the drug are proposed to occur via the C-7 and N-1 substituents of the quinolone (Palumbo *et al*, 1993). Both the Shen and Palumbo models, propose that quinolones bind to single stranded DNA after strand cleavage by DNA gyrase.

1.5.6 Other Binding Models

Several other quinolone binding models have been proposed, albeit with less supporting evidence. Yoshida *et al* (1993) proposed that quinolone binding would occur in the pocket of the DNA-gyrase complex that appears during the cleavage-reunion event and where quinolone binding is mediated equally by both *gyrA* and *gyrB* proteins. Current evidence does support the proposed interaction of the quinolone resistant DNA gyrase and the *gyrA* and *gyrB* proteins with the quinolones, as point mutations sustained within these genes have been shown to cause quinolone resistance (Yoshida *et al,* 1993; Willmott & Maxwell, 1993). Therefore, mutations

sustained within the *gyrA* and *gyrB* proteins can alter the optimal confirmation of the binding pocket resulting in reduced binding and a subsequent increase in resistance.

Another model proposed by Fan *et al* (1995) is based on the quinobenzoxazine model of binding. Quinobenzoxazines are a group of drugs that are structural analogues of norfloxacin, which possess no antibacterial activity but are able to exclusively inhibit mammalian topoisomerase II. The Fan (1995) model suggests that quinobenzoxazines bind to DNA in a magnesium dependent manner. However, this hypothesis is based on the drug binding to unwound duplex DNA and not to single stranded DNA and this has been shown not to be the case with bacterial topoisomerases (Shen & Pernet, 1985).

1.5.7 Quinolone Binding to Quinolone Resistant Mutants of DNA Gyrase

Mutations sustained within the A and B subunits of DNA gyrase have been shown to confer quinolone resistance on both intact bacteria and purified gyrase. Genetic changes associated with quinolone resistance have been found to occur most commonly at position 83 in the *E. coli gyrA*. In gram-positive bacteria, mutations shown to cause quinolone resistance occur at position 80 or 81, which is analogous to 83 in the *E. coli* GyrA protein. These quinolone resistance mutations occur in the amino terminal region of subunit A close to the active site of the enzyme (Tyr 122). Site directed mutagenesis of Serine 83 → Tryptophan in *E. coli* (Willmot & Maxwell, 1993) has been shown to significantly decrease norfloxacin binding to the DNA/DNA gyrase complex suggesting that the amino acid at position 83 interacts with the quinolone. However, other investigators have shown that similar mutations

of Serine83 → Leucine in *gyrA* and Lysine447 → Glutamic acid in *gyrB* binds to quinolones at the same levels as the wild type *gyrA* and *gyrB* in *E. coli* (Yoshida *et al*, 1993) although, the Aspartic acid 426 → Asparagine substitution in GyrB has been shown to bind lesser quinolone (Yoshida *et al*, 1993). The susceptibility to quinolones is compromised by mutations within the *gyrB* subunit at positions 426 and 447 in clinical isolates of *E. coli* (Yamagishi *et al,* 1986) thereby lending support to the proposed quinolone pocket model (Yoshida *et al* 1993). The suggestion that both GyrA and GyrB act *in concert* and mutations within the subunits can dictate a loss in binding and subsequent decrease in susceptibility to the fluoroquinolones is supported by clinical and laboratory evidence thereby securing a role for these enzymes in quinolone binding.

1.5.8 DNA Gyrase Quinolone Binding Models: A Critique

There is currently no scientific evidence to pinpoint the exact amino acid within DNA gyrase which is directly or indirectly involved in quinolone binding and which position and substitution within the quinolone pharmacore interacts with the enzyme. Both the Shen (1989) and Palumbo models (1993), propose that drug-DNA interactions occur either by hydrogen bonding or via the magnesium ion bridge between the C3 and C4 substituents and the DNA phosphate backbone. Investigations into the position of the Mg^{2+} ions during the binding event favour the latter hypothesis (Palu *et al*, 1992). Another position where the C3 and C4 substituents on the quinolone molecule can interact with the *gyrA* protein is Serine83. Single point mutations introduced at this position have been shown to reduce quinolone binding drastically (Willmot & Maxwell, 1993) and changes at this

position have been shown to decrease susceptibility to the quinolones in *E. coli* and other bacterial species (Hooper, 2000).

Both the Shen and Palumbo models suggest that the C7 position of the quinolone nucleus interacts with the DNA gyrase. Given the variability of modifications at this position, it seems an unlikely candidate for drug/DNA/gyrase interaction although it has been shown that substitutions at the C-7 position are able to alter intracellular interactions in *S. pneumoniae* (Alovera *et al*, 2000) and Yoshida *et al* (1993), have also found evidence that supports the involvement of the C7- piperazine substituents with subunit B of DNA gyrase in *E. coli*.

The Shen and Palumbo models suggest that binding occurs to single stranded DNA sites that are exposed after DNA cleavage by gyrase. Binding experiments performed with active site mutants bearing mutations at Tyr122 to either phenyalanine or serine in the *gyrA* protein have shown that binding to quinolone still occurs at levels consistent with the wild type enzyme (Critchlow & Maxwell, 1996). This result implies that DNA cleavage is not necessary for drug binding and questions the single stranded DNA binding prerequisite put forward by both models. Although, it is possible that a precleavage event occurs where a complex of gyrase, DNA, and the quinolone is formed prior to a more stable complex being established.

To further elucidate the mechanics of drug-binding, parallels may have to be drawn from the interactions of eukaryotic topoisomerase II and DNA. This model proposes the intercalation of the drug into the internucleotide space next to the cleaved phosphodiester bonds which in turn support the interaction with DNA gyrase residues (Freudenreich & Kreuzer, 1993). This interaction would be similar

regardless of the presence and absence of the cleavage event, although the presence of the quinolones may push the equilibrium towards cleavage. Modelling techniques support this proposal, where intercalation of the fluoroquinolones with DNA and DNA gyrase via the C6 and C7 position of the quinolones in the presence of magnesium ions has been observed (Llorente, 1996).

It is now accepted that quinolones bind to the DNA and DNA gyrase complex in the presence of magnesium ions however the exact mechanistic details involving quinolone interaction are vague and remain to be elucidated.

1.6 Mechanisms of Quinolone Resistance

When the fluoroquinolones were initially introduced into clinical therapy, resistance was considered to be a rare event. This was due to the apparent difficulty in raising laboratory mutants of *E. coli* (Smith, 1984) after quinolone challenge and the chromosomal mutations were found to be recessive to the wild type suggesting that interspecies transfer was unlikely. Despite these early optimistic predictions fluoroquinolone resistance has emerged in a number of clinically relevant species.

With the advent of rapid molecular techniques, quinolone mediated resistance has been mapped to mutations within a specific region called the quinolone resistance determining region (QRDR) within the target genes i.e. type II topoisomerases. Recently, fluoroquinolone resistance has also been found to be mediated through the hyper-expression of efflux pumps (Brenwald *et al*, 1998; Ubukata *et al*, 1989; Nikaido 1998). Other mechanisms of resistance such as enzymatic destruction of the fluoroquinolones were presumed to be unlikely as quinolones are totally synthetic

molecules and the presence of enzymes in nature to degrade these agents is therefore improbable. Plasmid mediated quinolone resistance was thought to be impossible because firstly certain resistance plasmids were found to increase quinolone susceptibility (Crumplin & Smith, 1981) and secondly, quinolones tended to eliminate plasmids (Hooper *et al*, 1984) by inhibiting both replication (Uhlin & Nordström, 1985) and transfer (Wiesser & Wiedemann, 1985). Apart from the inhibitory properties of the quinolones, the apparent absence of quinolone destroying enzymes and plasmid mediated resistance was attributed to the amount of quinolone usage. Today, quinolone usage accounts for 11% of all antibiotic prescriptions (Thomson, 1999) and thus, it is not surprising to note that the first instance of plasmid mediated fluoroquinolone resistance in a clinical isolate of *Klebsiella pneumoniae* has been identified (Martinez *et al*, 1998).

Quinolones are bactericidal antibiotics and exposure to these agents interferes with essential cellular processes resulting in cell death. Challenge with quinolones generally selects for resistance in pre-existing mutants within a bacterial population; however, it has now been shown that adaptive mutations can also appear after prolonged exposure to the antibiotic (Riesenfeld, 1997). This finding has far reaching consequences in a clinical setting where quinolones secreted from the body after a course of antibiotics could expose bacterial populations to low concentrations of the antibiotic, thereby priming the bacteria for the next antibiotic onslaught.

1.6.1 Target Site Modifications

1.6.1.1 Mutations in GyrA

It has now been accepted that fluoroquinolones attack bacteria by inhibiting the action of the type II topoisomerases within the cell. Gellert *et al* (1976) made the first association that quinolones target bacterial DNA gyrase by demonstrating the inhibition of DNA gyrase with nalidixic acid. The first molecular characterisation of a quinolone resistance mutation in *gyrA* was reported by Yoshida *et al* (1988). Subsequent cloning and sequencing of the *gyrA* gene from the quinolone resistant bacteria found mutations in the N-terminus region of the GyrA polypeptide close to the tyrosine residue 122, which is covalently bound to DNA during DNA cleavage (Yoshida *et al*, 1988). In *E. coli*, fluoroquinolone resistance associated mutations were found to consistently occur within the same region spanning from codon 67 to 106 which has since been termed the QRDR (Piddock, 1999). This region has been found to be highly conserved across different bacterial species. In *S. pneumoniae* mutations within the homologous region have also been associated with quinolone resistance (Balas *et al*, 1998; Pan & Fisher, 1996a). In *E. coli*, changes within the QRDR have been found to occur most frequently at Serine 83 which is altered to either Leucine or Tryptophan resulting in a 32-fold increase in resistance to ciprofloxacin (Yoshida *et al*, 1993; Yoshida *et al*, 1988). Other positions within the QRDR of the *gyrA* gene have also been associated with quinolone resistance, however, in comparison to the change that occurs at position 83 the decreases in susceptibility are not as pronounced. Mutations at position 83 have been shown to co-exist with other mutations at position 87 within the *gyrA* QRDR of *E. coli*. In

S. pneumoniae, the changes that occur at *gyrA* are similar to *E. coli* except that the analogous region to Serine 83 is mapped at position 80 within the pneumococcal genome (Balas *et al*, 1998). Mutations, which occur at position 81, are usually substitutions from serine to phenyalanine or tyrosine (Janoir *et al*, 1996; Gootz *et al*, 1996). Certain mutations in *E. coli* like Glycine81 to Aspartate or Glutamic acid 106 to Arginine in *gyrA* have been found to confer increased resistance to the fluoroquinolones rather than nalidixic acid (Moniot-Ville *et al*, 1991). In *S. pneumoniae*, *gyrA* mutations have been found to result in increased resistance to the broad-spectrum quinolones (sparfloxacin, moxifloxacin) rather than the older agents (ciprofloxacin) (Fukuda & Hiramatsu, 1999). Double *gyrA* mutations sustained within the QRDR of *E. coli and K. pneumoniae* are usually at positions 83 and 87 and can coexist resulting in high-level resistance (Weigel *et al*, 1998; Deguchi *et al*, 1997). In contrast, both clinical and laboratory isolates of *S. pneumoniae* have been found to sustain single but not double *gyrA* mutations (Jones *et al*, 2000; Bast *et al*, 2000a; Pan & Fisher, 1996a; Pan & Fisher, 1997; Pan & Fisher, 1998).

1.6.1.2 Mutations in GyrB

The first description of mutations sustained within GyrB was reported by Yamagishi *et al* (1986) when nalidixic acid resistant mutants of *E. coli* were analysed for mutations. Two mutants sustained changes within *gyrB* at Asp426 → Asn and Lys447 → Glu, resulting in resistance to acidic quinolones such as nalidixic acid (MIC = 32–128mg/l). However, the mutant with the Lys447 → Glu, mutation was found to be 4-fold more susceptible than the wild type to fluoroquinolones like

ciprofloxacin. GyrB mutations associated with fluoroquinolone resistance have also been reported in quinolone resistant *Ps. aeruginosa* (Yoshida *et al*, 1990a) and *N. gonorrhoea* (Deguchi *et al*, 1996). In gram-positive bacteria, *S. aureus* and *S. pneumoniae* have been found to sustain mutations in *gyrB* related to quinolone resistance. In fluoroquinolone resistant laboratory mutants of *S. aureus* (Schmitz *et al*, 1998a) and *S. pneumoniae* (Pan & Fisher, 1998) mutations within *gyrB* at Asp437 → Asn, Arg458 → Gln, and Glu474 → Lys respectively, were identified defining the presence of an analogous region in gram-positive bacteria.

1.6.1.3 Occurrence of *gyrA* and *gyrB* Mutations

In gram-negative bacteria, the occurrence of *gyrB* mutations have been found to be relatively lower than *gyrA* mutations. A survey of 16 clinical isolates of *E. coli* identified that 15 of the isolates sustained changes within *gyrA* and 1 isolate with a change in *gyrB* (Vila *et al*, 1994). In addition, another study showed that when 18 laboratory mutants of *E. coli* selected with norfloxacin and lomefloxacin, no mutants with *gyrB* mutations were identified (Tavio *et al*, 1999). Therefore, it appears that in laboratory selected mutants the frequency of mutations in *gyrA* is clearly higher than *gyrB* mutations. This is also represented in the clinical situation where there is a clear predominance of *gyrA* mutations over *gyrB* changes in conferring clinical levels of fluoroquinolone resistance. The minimal contribution of *gyrB* mutations in the development of fluoroquinolone resistance is also represented in other gram-negative species like *Ps. aeruginosa* (Yoshida *et al*, 1990a) and *A. baumanii* (Vila *et al*, 1997).

In gram-positive bacteria, like *S. aureus*, *gyrB* mutations are thought to act in concert with *gyrA* mutations to confer fluoroquinolone resistance, although, a survey done with 116 clinical isolates showed that mutations sustained within *gyrB* were silent with no resulting amino acid changes (Schmitz *et al,* 1998a). In *S. pneumoniae*, *gyrB* changes of Asp435 → Asn and Glu474 → Lys identified in laboratory mutants selected with ciprofloxacin (Pan & Fisher, 1996a) and clinafloxacin (Pan & Fisher, 1998) were also associated with quinolone resistance. In both sets of mutation studies, only one mutant sustained the change in *gyrB*. With the clinafloxacin mutation study, the *gyrB* mutation of Glu474 → Lys was the only change identified in that clone in contrast, to the *gyrB* mutation (Asp435 → Asn) observed within the ciprofloxacin selected mutant where the change was sustained together with either *gyrA* or *parC* substitutions. A survey done with clinical isolates of *S. pneumoniae* indicated that none of the isolates sustained a mutation within *gyrB* (Jorgensen *et al*, 1999) although another study by Heaton *et al* (1999) has identified the Glu474 → Lys change in combination with a *parE* and *parC* mutation in two clinical pneumococcal isolates.

The apparent rarity of single *gyrB* mutations within both clinical and laboratory mutants indicates a secondary contributory role of these changes towards the development of fluoroquinolone resistance. Thus, it does appear that *gyrA* mutations play a bigger role in resistance development in both gram-negative and gram-positive bacteria.

1.6.1.4 Mutations in ParC

Enzyme inhibition studies done on the *E. coli* topoisomerase IV enzyme, found that 30 times more antibiotic was required to inhibit topoisomerase IV rather than DNA gyrase in *E. coli* (Kato *et al*, 1990). Studies by Hoshino *et al* (1994) demonstrated that fluoroquinolones were able to exert considerable activity against topoisomerase IV mediated catenation particularly in *gyrA* mutants. These observations clearly indicate a role for topoisomerase IV as a secondary target for the fluoroquinolones in gram-negative bacteria in the absence of a sensitive *gyrA* and contribute significantly to the decrease in susceptibility to these agents. This phenomenon is seen with the other gram-negative species like *K. pneumoniae* (Weigel *et al*, 1999), *Ps. aeruginosa* (Yoshida *et al*, 1990a) and *N. gonorrhoeae* (Deguchi *et al*, 1996).

In gram-positive bacteria, topoisomerase IV (Ferrero *et al*, 1995) was presumed to be the primary fluoroquinolone target as initial *in vitro* mutation studies with *S. aureus* demonstrated that mutants sustained changes within *parC* prior to *gyrA*. Mutants sustaining both *parC* and *gyrA* changes were found to exhibit the least susceptibility to ciprofloxacin. The changes associated with the decrease in fluoroquinolone susceptibility in *parC* are situated at position 80 analogous to Serine83 within the *E. coli gyrA*.

Other gram-positive species like *S. pneumoniae* (Pan & Fisher, 1996a) and *E. faecalis* (Kanematsu *et al*, 1998) were also found to sustain mutations within *parC* after ciprofloxacin challenge lending to support to the assumption that topoisomerase IV was the primary target of quinolones in gram-positive bacteria.

However, subsequent *in vitro* studies involving *S. pneumoniae* and fluoroquinolones such as sparfloxacin (Pan & Fisher, 1997), gatifloxacin (Fukuda & Hiramatsu, 1999) and gemifloxacin (Heaton *et al*, 1999) have demonstrated that the mutants harbour mutations within *gyrA* prior to changes within *parC*. In *S. pneumoniae*, the mutations that occur within *parC* (Ser79 → Tyr) confer low to intermediate levels (i.e. 8mg/l) of resistance but when coupled with a *gyrA* mutation, the decrease in susceptibility is 8-fold higher (Pan *et al*, 1996a; Fukuda & Hiramatsu, 1999; Heaton *et al*, 1999).

1.6.1.5 Mutations in ParE

Only recently have mutations within the *parE* subunit been associated with quinolone resistance. Mutation studies involving *S. pneumoniae* challenged with ciprofloxacin (Pan & Fisher, 1996a) and clinafloxacin (Pan & Fisher, 1998) have shown that none of the ciprofloxacin selected mutants sustained a *parE* mutation although only one clinafloxacin mutant harboured a change of Pro454 → Ser. Studies involving both clinical isolates and laboratory mutants of *S. pneumoniae* indicate that changes within the *parE* subunit confers low level resistance to the quinolones and are usually present in combination with either a *parC* or *gyrA* mutation (Jorgensen *et al*, 1999). In contrast, a survey done with 20 quinolone resistant isolates of *E. coli* found none of the isolates sustained a mutation in *parE* (Ruiz *et al*, 1997).

1.6.1.6 Occurrence of *parC* and *parE* Mutations

In clinical isolates of *S. pneumoniae*, changes within *parC* precede *gyrA* changes resulting in high-level fluoroquinolone resistance. Changes within *parE* are found in

clinical isolates albeit, at a much lower frequency than *parC*, where *parE* changes on its own or in combination with a *parC* mutation contribute to low to intermediate levels of fluoroquinolone resistance (Jones *et al*, 2000; Bast *et al*, 2000a; Perichon *et al,* 1997). In gram-negative bacteria, studies have shown no evidence of the involvement of *parE* mutations within clinical isolates with respect to fluoroquinolone resistance and the prevalence of these changes within laboratory mutants is largely unknown. Clinical and laboratory mutants of *E. coli* demonstrate that *parC* changes are associated with contributing to higher levels of resistance to the fluoroquinolones and are always found in combination with *gyrA* mutations (Weigel *et al*, 1998; Tavio *et al*, 1999).

1.6.1.7 Primary and Secondary Quinolone Targets

Controversy surrounds the debate about the targets of quinolone action within gram-positive bacteria. In gram-negative bacteria, consistent changes within the QRDR of *gyrA* with quinolone challenge supports the assumption that *gyrA* is the primary target (Tavio *et al,* 1999). In contrast, in gram-positive bacteria particularly, *S. pneumoniae* this does not appear to be the case. Stepwise selection of *S. pneumoniae* with various quinolones have shown different patterns of mutation development (See Table 1.2).

Drug	Primary Target	Secondary Target
Ciprofloxacin‡	ParC/ParE	GyrA
Sparfloxacin	GyrA	ParC
Trovafloxacin	ParC	GyrA
Clinafloxacin‡‡	GyrA + ParC	—
Gatifloxacin	GyrA	ParC
Gemifloxacin	GyrA	ParC

Table 1.2: Targets in *S. pneumoniae* after Quinolone Challenge

‡ = some *in vitro* mutation studies have shown changes in the ParE subunit as well as the ParC.
‡‡ = Clinafloxacin selection induces changes in both *gyrA* and *parC* subunits.

It has been shown that mutants selected with ciprofloxacin and trovafloxacin sustaining changes within topoisomerase IV (ParC) show no resistance to sparfloxacin and gatifloxacin but exhibit cross resistance to one another. This indicates that *parC* is the likely target of ciprofloxacin (Fukuda & Hiramatsu, 1999; Pan & Fisher, 1996a) and trovafloxacin (Gootz *et al*, 1996). In contrast, mutants selected with sparfloxacin, gatifloxacin and gemifloxacin exhibited no cross resistance to the other quinolones except to each other (Fukuda & Hiramatsu, 1999). This observation corresponds to that made in *S. aureus* where mutations that confer resistance to one quinolone do not necessarily confer resistance to other quinolones (Takahashi *et al*, 1998, Durham, 2000). Clinafloxacin is unique in its ability to select for mutations in both targets (Pan & Fisher, 1998).

Therefore, it is tempting to speculate that structural modifications of the aforementioned quinolones are able to dictate the choice of target within *S. pneumoniae*. In fact, studies now show that the addition of a benzenesulfonylamido group to the C-7 piperazinyl ring of ciprofloxacin markedly

affects potency in *S. pneumoniae* and more importantly changes the specificity of the quinolone target from topoisomerase IV to DNA gyrase A (Alovero *et al*, 2000).

Enzyme inhibitory studies of fluoroquinolones on recombinant proteins of pneumococcal type II topoisomerases (ParC and GyrA) reveal discrepancies with the *in vitro* data presented in Table 1.2. As it has been demonstrated that the concentration of fluoroquinolones, like ciprofloxacin and sparfloxacin, required to inhibit wild–type topoisomerase IV were 8–16 times lower than that required to inhibit wild type DNA gyrase (Morrissey & George, 2000) implying that topoisomerase IV is the genuine target of quinolones like ciprofloxacin and sparfloxacin. Further studies done with ciprofloxacin, sparfloxacin and clinafloxacin, which have differential activity against the pneumococcal topoisomerases, demonstrated that all the quinolones tested preferentially inhibited topoisomerase IV rather than *gyrA* (Pan & Fisher, 1999). This result also concurs with inhibitory studies performed with staphylococcal topoisomerase proteins (Blanche *et al*, 1996). So why the discrepancy? One reason for the differential activities that are observed with cell bound topoisomerases and purified enzymes could be due to the failure of conditions within the enzyme assays to simulate the *in vivo* conditions like the presence of a specific DNA template, Mg^{2+}ions, polyamine, and ATP concentrations. A further point is the possibility of mutations outwith the QRDR which may not be detected by the techniques used to determine the mutations for the *in vitro* studies, but may manifest itself when the entire topoisomerase is purified and exposed to the quinolone.

Does the topology of the topoisomerases differ between both gram–negative and positive bacteria? In fact the cloning and characterisation of the pneumococcal *gyrA* (Balas *et al*, 1998) has illustrated differences to that of the *E. coli gyrA*. The extended –10 sites that occur within the promoter box in the pneumococcus (Sabelnikov *et al*, 1995) result in overexpression of *gyrA* when expressed in other hosts, therefore the reproducibility of the exact function and properties of the native pneumococcal protein is lost. Finally, it is possible that quinolones may be unevenly distributed within the bacterial cell thereby affecting complex formation with the topoisomerases differently. This could be important if the enzymes themselves are distributed in a non–uniform manner as seen with *B. subtilis* where topoisomerase IV has a bipolar localisation and gyrase is associated with a nucleoid (Huang *et al*, 1998). In *S. pneumoniae*, the intracellular positions of both topoisomerase IV and DNA gyrase have not been determined. It is apparent from these points that neither mutation experiments or enzyme assays will independently answer the question of quinolone targets within *S. pneumoniae*.

1.6.1.8 Efflux Pumps

Efflux pumps are ubiquitous components of the bacterial cell walls that allow them to be a formidable factor in antimicrobial resistance (Levy, 1992). The efflux of cytotoxic agents is defined as an active, unidirectional export of compounds from the bacterial cytoplasm into the external medium. This transport is usually protein-mediated and performed by a uniport mechanism coupled to ATP hydrolysis or by an antiport mechanism in which drug efflux is linked to proton influx i.e. energised by the proton motive force (PMF) (Paulsen *et al*, 1996).

Efflux pumps can be classified under four main groups based upon sequence homology, molecular architecture and mechanism of action. The five main groups are (i) ATP Binding cassette (ABC) Transporters, Staphylococcal (or Small) Multidrug resistance (SMR), Resistance Nodulation Division (RND), Major Facilitator (MF) and the recently described multi-drug and toxic compound extrusion family (MATE) (Levy, 1992; Nikaido, 1998; Brown *et al*, 1999). These pumps are broad spectrum, which allows them to efflux a range of toxic substances including antimicrobial compounds (reviewed by Poole, 2000).

Efflux pumps are thought to perform a dual action. Two schools of thought exist, in the first where pumps are thought to perform a physiological function and incidentally pump out antimicrobial agents and in the second where the pumps are purely present to combat antibiotic challenge. Initial studies involving knock out multi-drug efflux pump mutants demonstrated, no obvious deficiencies in physiological function except for an increase in antibiotic susceptibility (Neyfakh, 1997). Investigations into the evolution of these pumps indicates that multi-drug transporters do not share common homology even within the superfamilies but do so with substrate-specific transporters. This indicates that multi-drug transporters may possess a dual function by promoting the efflux of both specific metabolites and cytotoxic drugs (Neyfakh, 1997).

Both gram-positive and gram-negative bacteria possess cytoplasmic barriers to reduce the transport of toxins into the cell. However, gram-negative bacteria possess an outer membrane with specialised porins that limit cellular transport to hydrophilic rather than hydrophobic molecules. Therefore, in gram-negative bacteria a dual

cellular barrier has to be crossed prior to gaining entry into the cell proper, unlike in gram-positive bacteria, where only one barrier needs to be breached. The assembly of efflux pumps mirror these differences and structurally the gram-negative efflux pumps are more complex (See Figure 1.11).

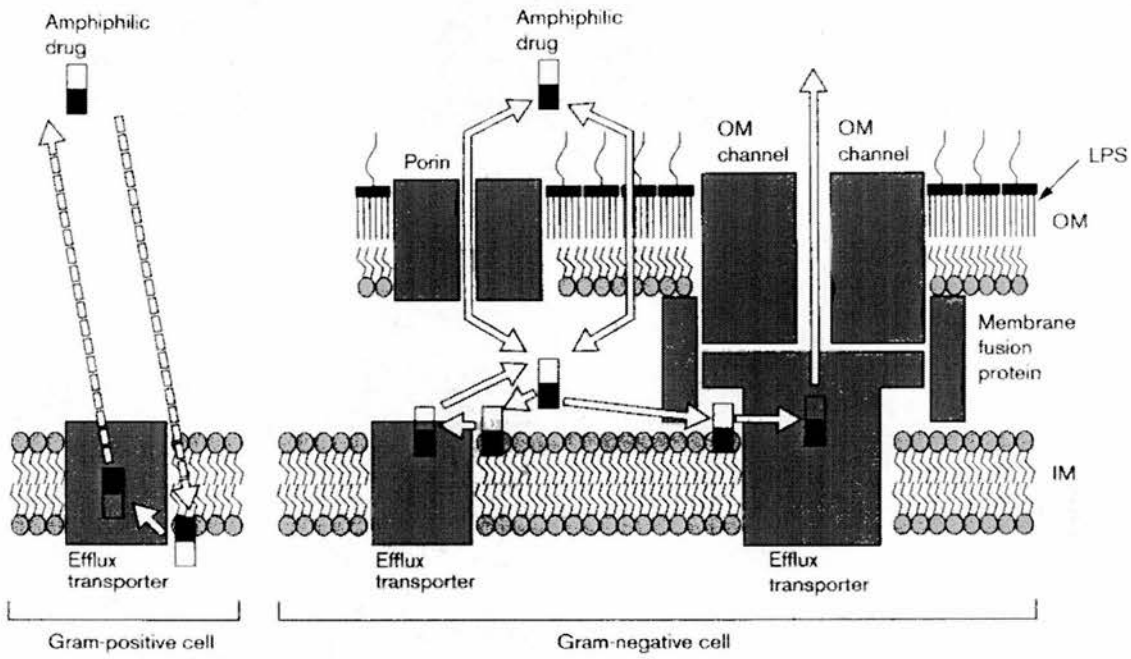


Figure 1.11: Variable Configurations of Bacterial Efflux Pumps
(Taken from Nikaido, 1998)

Bacterial multidrug efflux pumps exist in two different configurations within gram-negative and positive bacteria. In the gram-positive cell (left), drugs diffuse in unhindered and are pumped out (dashed arrows). In gram-negative bacteria, drugs diffuse through the outer membrane via specialised porins and are extruded within the periplasmic space (solid arrows). Drug molecules are captured within the bilayer although this may not be the mechanism with all families of transporters. Complex efflux machinery only occurs in gram-negative bacteria although drug efflux from the cytoplasm occurs in both species.
LPS = lipopolysaccharide
OM = outer membrane
IM = inner membrane

The Resistance Nodulation division (RND) and multi-drug and toxic compound extrusion (MATE) group of efflux pumps seem to be restricted to gram-negative bacteria (reviewed by Poole, 2000). The RND pumps possess translocases (12-transmembrane alpha helices and are believed to act as monomers) and these

pumps occur *in concert* with a linker protein and outer membrane channel. The AcrAB–TolC *E. coli* pump and the MexAB–OprM of *Ps. aeruginosa* are examples (Nikaido, 1998). The substrate profile of the RND family of pumps is diverse and includes antibiotics, dyes, and detergents (Nikaido, 1998). Homologs of the RND pump have been identified in other gram-negative species such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. Apart from possibly providing an initial defence against antibiotic challenge, the hyper-expression of efflux pumps can also manifest a multi-drug resistant phenotype.

The initial investigations of efflux pumps in gram-positive bacteria were limited to *Staphylococcus aureus*. The pumps, *qacA* and *smr* identified in this organism were found to be plasmid mediated (Nikaido, 1998). However, plasmid mediated efflux pumps cannot be considered to be the sole contributory factor for the reduction in susceptibility as plasmids generally harbour other resistance genes. The majority of gram-positive efflux pumps are chromosomal in origin and tend to be classified under the following groups: SMR (Small multi-drug Resistance), ABC (ATP-binding cassette) or MFS (Major Facilitator Superfamily) group and are energised by ATP hydrolysis or proton motive force (PMF) (Paulsen *et al*, 1996). With the SMR and MFS multi-drug transporters, the range of compounds that are pumped tends to be limited as in the case of *qacA* (cationic dyes, quaternary amines, and fluoroquinolones) (Rouch *et al*, 1990) (See Figure 1.12). *Blt,* a pump identified in *Bacillus subtilis* exports out spermidine (a natural polyamine) and is postulated to be simultaneously involved in the physiological efflux of polyamines (Neyfakh, 1997). It is recognised as a possible multi-drug efflux pump as there may be certain cationic antibiotic drugs that mimic the structures of polyamines. This observation supports

the hypothesis that efflux pumps are part of the physiological machinery of the bacterial cell.

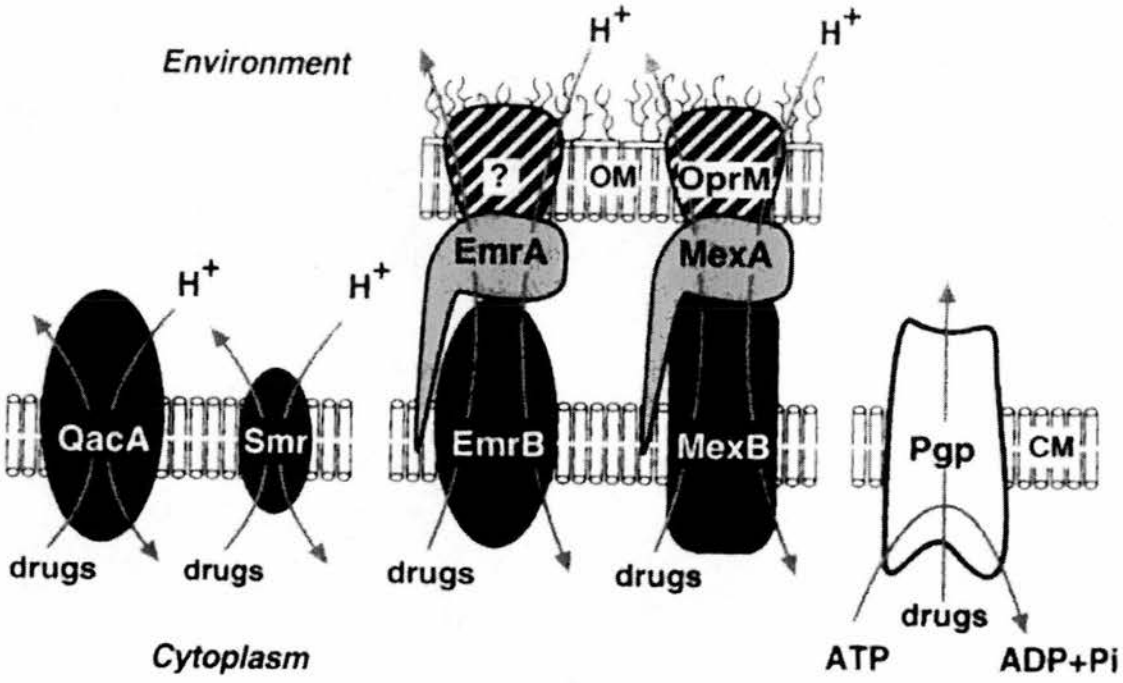


Figure 1.12: Diagrammatic Representation of Efflux Pumps
(Taken from Paulsen *et al*, 1996)

Diagrammatic representation of efflux pumps found in bacteria. The *Staphylococcus aureus* QacA, EmrB and Smr proteins. The *Pseudomonas aeruginosa* MexB proteins utilise the proton gradient for energy. In contrast, the multi-drug efflux pump P-gycoprotein is driven by ATP hydrolysis.

The ABC transporters are different as they mimic the human P-glycoprotein in its wide substrate specificity. The other gram-positive pumps *LmrA* and the MFS pump *LmrP*, have been shown to capture their substrates from within the bilipid layer (inner leaflet of cytoplasm) (Bolhuis *et al*, 1996). The MFS group of membrane transporter proteins have been found to be present in bacteria and higher eukaryotes and have been divided into five distinct function clusters involved in (i) drug resistance (ii) sugar uptake (iii) uptake of Krebs cycles intermediates (iv) phosphate ester/phosphate antiport and (v) oligosacharide uptake (Marger & Saier, 1993). Examples of the proton motive force dependent 12 transmembrane segment drug

efflux proteins include the streptococcal efflux pump PmrA (Gill *et al*, 1999), *B. subtilis* pump BmrA (Neyfakh *et al*, 1991) and the staphylococcal pump NorA (Yoshida *et al*, 1990). PmrA, BmrA and NorA all possess a similar substrate range i.e. acridine orange, ethidium bromide and fluoroquinolones such as norfloxacin and ciprofloxacin (Brenwald *et al*, 1997; Neyfakh *et al*, 1991; Yoshida *et al*, 1990b). Sequence alignment of *pmrA* with other gram-positive pumps such as BmrA and NorA has identified 24% homology (Gill *et al*, 1999). Transmembrane segment prediction of PmrA has also indicated the presence of 12TMSs, which indicates similarity to other 12-TMS proton dependent pumps such as NorA (Paulsen *et al,* 1996). It has been shown that drug transport and resistance mediated by NorA, BmrA and PmrA is inhibited by the mammalian P-glycoprotein pump inhibitor reserpine (Neyfakh *et al*, 1991) which allows the phenotypic analysis of these gram-positive efflux pumps (Neyfakh *et al*, 1991). If an efflux pump were expressed, a reduction would be observed with the antibiotic and reserpine combination to the antibiotic only. However, the shortfall of this technique is that a reduction may only be observed with pumps, that are reserpine sensitive.

1.6.1.9 Contribution of Efflux Pump Expression to Fluoroquinolone Resistance

Two independent studies investigating the expression of the efflux pump *pmrA* in clinical streptococcal isolates have shown that increased efflux expression is the most prevalent mechanism of resistance to the fluoroquinolones (Brenwald, *et al,* 1998; Morrissey *et al*, 1999). It has been demonstrated that inhibition of the *pmrA* pump increases susceptibility to ciprofloxacin, norfloxacin (Gill *et al*, 1999) and ofloxacin (Broskey *et al*, 2000). Reserpine inhibition has been shown to suppress the *in vitro*

emergence of ciprofloxacin resistance in *S. pneumoniae* clearly indicating that efflux mediated mechanisms play a role in fluoroquinolone resistance (Markham, 1999). PmrA does not appear to have any effect on the newer generation hydrophobic quinolones such as grepafloxacin (Morrissey *et al*, 1999) and gemifloxacin (Heaton *et al*, 1999, Broskey *et al* 2000) indicating that reserpine potentiation of the fluoroquinolone sensitivity is restricted to the older agents.

In *S. aureus*, inhibition of the efflux pump NorA with reserpine has been found to increase the level of susceptibility to ciprofloxacin by four-fold (Neyfakh *et al*, 1991). NorA and PmrA, exhibit similar substrate ranges and have been shown to efflux some hydrophilic quinolones such as ciprofloxacin and appear not to export other compounds like moxifloxacin (reviewed by Poole, 2000). In *S. aureus*, the increased efflux of quinolones has been attributed to either the overexpression of the wild type gene (Kaatz & Seo, 1995) or a single nucleotide change within the NorA promoter (Ng *et al*, 1994). Mutations resulting in altered substrate specificities of the BmrA and NorA pumps have been found mainly within the C-terminal regions of these proteins (Griffith *et al*, 1992). Fluoroquinolone resistance mediated by the streptococcal efflux pump PmrA is thought to be the hyperexpression of the pump as it has been detected in fluoroquinolone susceptible strains. Unlike NorA and BmrA, which are induced by the substrates they efflux (Paulsen, 1996), the exact inducer of *pmrA* has not been deduced although the regulator gene mtrA has been identified (Gill *et al*, 1999).

In gram-negative bacteria, like *Ps. aeruginosa* fluoroquinolones have been found to select for efflux type mutants (Köhler *et al*, 1997) prior to topoisomerase mutations.

This trend also extends to ß-lactam drugs, where a study of carbenicillin resistant *Ps. aeruginosa* show that almost 80% of the strains did not produce carbenicillin–hydrolysing ß-lactamases but exhibited elevated efflux (Bert *et al*, 1996). Thus, it does appear that in both gram-positive and gram-negative bacteria, the expression of the efflux pump precedes the presence of target mutations, which could imply that efflux mutations are precursors in the development of resistance. However, efflux has not been characterised as a mechanism of resistance for all antibiotics thereby questioning the specific conditions that could favour the selection of efflux mutants. One factor appears to be the reduced activity of antibiotics against the target bacteria thereby inducing the efflux phenotype as observed with the carbenicillin resistant *Ps. aeruginosa* and quinolone resistant *S. aureus* and *S. pneumoniae* (Nikaido, 1998; Brenwald *et al*, 1998). Secondly, the absence of conventional resistance mechanisms against antibiotics such as the fluoroquinolones and carbenicillin, has encouraged the emergence of efflux mutants.

Efflux pumps are present in bacteria and their role in the development of antibiotic resistance is still being established. It is interesting to note that the prevalence and expression of efflux pumps are most frequent in bacteria like *Ps. aeruginosa*, *S. aureus* and *S. pneumoniae* that are only moderately susceptible to some antibiotics. This adaptability demands different treatment options through the design and development of efflux inhibitors.

1.6.2 Other Mechanisms of Resistance

1.6.2.1 Plasmid Mediated Resistance

The first confirmed report of plasmid mediated quinolone resistance was from a clinical isolate of *K. pneumoniae* (Martinez *et al*, 1998). This multi-drug resistance plasmid was found to harbour resistance to aztreonam, ceftazidime, kanamycin, gentamicin, tobramycin, trimethoprim, mercuric chloride and ciprofloxacin and conferred reduced susceptibilities to these agents when transferred. However, clinical levels of resistance were only observed when the plasmid was transferred into an outer membrane deficient strain. Although, the possibility for plasmid mediated fluoroquinolone resistance to become a clinical reality may exist, no further reports of plasmid mediated quinolone resistance have been noted.

1.6.2.2 Adaptive Mutations

Adaptive mutations arise through prolonged antibiotic exposure in non-dividing or slowly dividing cells. Adaptive mutations also give rise to phenotypes, which allow cell growth with no fitness cost. This effectively implies that adaptive mutations allow growth inhibited bacteria to resume growth after the antibiotic pressure is removed. This phenomenon has been characterised in *E. coli* MG1655 cells after exposure to ciprofloxacin over a 7 day period (Riesenfeld *et al*, 1997). Although quinolones are bactericidal, they nurture a population that may be described as being non culturable but viable. Clinically, the implications of this mechanism are enormous as cessation of antibiotic treatment may encourage the emergence of resistant bacterial cells. The actual prevalence of this mechanism in *in vivo*

conditions and in other species is largely unknown. It remains to be seen whether the newer bactericidal quinolones are able to induce such mutations. Thus, the changes associated with adaptive mutations may allow the prolonged survival of bacterial cells leading to resistance development.

Both plasmid mediated resistance and adaptive mutations have only recently been characterised in gram-negative bacteria and the extent of these mechanisms are still largely unknown in gram-positive bacteria.

1.6.2.3 Horizontal Transfer of Fluoroquinolone Resistance Determinants

In naturally transformable *S. pneumoniae*, resistance to the β-lactam antibiotics has been attributed to penicillin-binding protein alterations resulting from genetic exchanges with commensal viridans streptococci of the oral flora (Tomasz, 1997; Sibold *et al*, 1994). Recently, studies describing the emergence of fluoroquinolone resistance in viridans streptococci have been documented (Yan *et al*, 2000; Fernandiz *et al*, 1999; Gonzalez *et al*, 1998) questioning the possibility of interspecies transfer of altered genes mediating quinolone resistance. In a study done by Janoir *et al* (1999) it was found that high-level fluoroquinolone resistant pneumococcal transformants were obtained at low frequencies when a double ParC-GyrA quinolone resistant mutant of *S. mitis* was used as a donor. Analysis of clinical isolates of both *S. pneumoniae* and viridans streptococci support the possibility of horizontal transfer of resistant topoisomerase genes (Fernandiz *et al*, 2000) although only a small number of isolates were investigated. Statistically, the clinical occurrence of this event has been determined to occur at <1% (Bast *et al*, 2000b) and would require the presence of highly competent cells for DNA uptake.

1.7 Respiratory Tract Infections

Community and hospital acquired respiratory tract infections are a major public health issue and these infections are the predominant indication for antibacterial prescribing in both the community and hospital (Finch, 1995). Respiratory infections are caused by a multitude of bacterial species however, pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* have been implicated more frequently than others. This observation is validated by studies that have demonstrated the presence of these pathogens in acute exacerbation of acute bronchitis (AECB) and community acquired pneumonia (CAP), a common cause of hospital and community consultations (Moine *et al*, 1994). The status of these pathogens in causing severe infections in both the community and hospital, has enormous implications in their control and treatment.

1.7.1 The Pneumococcal Problem

S. pneumoniae was first described by Klebs in 1875 (reviewed by Austrian, 1981). It has been known for more than 100 years as the most important bacterial pathogen of respiratory tract infections in adults and children (Harwell & Brown, 2000). *S. pneumoniae* is a resident of the upper respiratory tract in healthy individuals and at any one given time up to 60% of people in a community can carry this bacterium in the mucosal epithelium of the nasopharynx (Johnston, 1991). However, it has been observed that most infections do not occur after prolonged carriage but follow on after the acquisition of different serotypes (Alonso de Velasco *et al*, 1995). This observation suggests that the immune status of the host at the moment of colonisation and the pathogenic potential of the infecting strain, determines whether the bacterium

remains a non-infectious resident of the nasopharynx or becomes invasive (Johnston, 1991). *S. pneumoniae* causes a variety of other infections which include meningitis, sinusitis, osteomyelitis, otitis media, bronchitis and soft tissue infections (Tuomanen *et al*, 1995).

1.7.1.1 Virulence Factors

The exact mechanism of pneumococcal pathogenesis is still largely unknown although it is thought to be mediated by the immune cascade reaction (Tuomanen *et al*, 1995). This cascade results in extensive inflammation, which presents itself as a complete disruption of the bronchial epithelium and its protective function. This extensive cellular damage and its subsequent complications are responsible for the morbidity and mortality associated with pneumococcal infections (Alonso de Velasco *et al*, 1995).

1.7.1.2 Capsule Polysaccharide

Since the beginning, the polysaccharide capsule has long been recognised to be the principal virulence determinant of the pneumococcus. Eighty-four distinct serotypes have been identified according to the antigenic compositions of the capsules. Serotyping of these capsules can be done by the Quellung reaction, where specific antibodies react with capsular antigens on the capsular surface of the organism. More than 80% of pneumococcal infections are caused by 23 of the 84 distinct serotypes (Tuomanen *et al*, 1995). Encapsulated strains were found to be at least 10^5 times more virulent than strains lacking the capsule (Johnston, 1991). Despite, being attributed to the pathogenic potential of the bacterium, the capsule itself is non-toxic

in its pure form. However, it seems to enhance virulence by protecting the bacterium from ingestion by the host's phagocytes (Tuomanen *et al*, 1995; Finland *et al*, 1932).

1.7.1.3 Cell Wall and Cell Wall Polysaacharide

There is no evidence to support the role of the by-products and degradation of the pneumococcus in the clinical manifestation of pneumococcal disease. Although, constituents of the cell wall particularly the phosphorylcholine residue of the C-polysaacharide have been shown to induce the inflammatory cascade by inducing antibody response and complement activation (Johnston 1991).

1.7.1.4 Pneumococcal Protein Antigens and Toxins

Various proteins have been implicated in pneumococcal pathogenicity, although only a few have been proven to be genuine virulence factors. One of the enzymes produced by *S. pneumoniae* is neuraminadase, which facilitates the attachment of the bacteria to the epithelial cells by cleaving sialic acid from the host glycolipids and gangliosides. Partially purified neuraminadase has been shown to induce meningitis-like symptoms in mice, although this has been disputed due to possible contamination of the neuraminadase preparations (Alonso de Velasco *et al*, 1995).

Pneumolysin is an intracellular protein that belongs to the family of thiol-activated toxins. This enzyme is not secreted by the pneumococcus but released during autolysis. At high concentrations, pneumolysin forms oligomers on mammalian cell membranes, giving rise to transmembrane pores that result in cell lysis (Boulnois, 1992). Pneumolysin is also known to stimulate the production of inflammatory cytokines and complement activation (Alonso de Velasco *et al*, 1995). This

inevitably results in extensive cellular damage of the bronchial epithelium disrupting functions like cilia beating and the migration of neutrophils. Pneumolysin release has been shown to mediate the effects of autolysin. Autolysin-deficient strains are less virulent than autolysin producing variants and immunisation with autolysin confers some protection in mice (Alonso de Velasco *et al*, 1995; Watson *et al*, 1995).

Pneumococcal surface protein A is associated with the structural and antigenic variability between different pneumococcal strains and is found in most of the clinical isolates of *Streptococcus pneumoniae.* The exact functions of this surface protein are still unknown however, the introduction of surface protein A deficient strains into experimental animals, has been shown to reduce virulence and prolong survival (Alonso de Velasco *et al*, 1995).

There are still many questions pertaining to the molecular mechanisms of pathogenesis associated with pneumococcal infections. The recent discovery of hydrogen peroxide production by the pneumococcus and its toxic effects on rat alveolar epithelium (Johnston, 1991) introduces a new component to the pathogenesis puzzle. Suffice it to say, that many aspects of the host-bacterium relationship are still unknown.

1.7.1.5 Epidemiology and Risk Factors for Colonisation

Mortality associated with bacteriaemic pneumonia remains at 25% and has remained unchanged for over 40 years despite advances in antimicrobial chemotherapy (Kramer *et al*, 1987). In the UK alone, the pneumococcus is responsible for 30–50% of community acquired pneumonia and 8% of nosocomial pneumonia (Obaro *et al*,

1996). It is also a major factor in chronic bronchitis affecting more than 1 million individuals and is responsible for 5% of all deaths. Globally, the pneumococcus accounts for 1 million deaths each year in children under the age of 5 years (Leowski, 1986). The initial introduction and use of penicillin against pneumococcal pneumonia reduced the fatality rate of the disease from 30% to as low as 5%. Predisposing risk factors have been found to include age, social status, immunocompetence and correspondingly pneumonia has been found to be a major cause of mortality either in the very young or old (Obaro *et al*, 1996).

1.7.1.6 Antibiotic Resistance in *S. pneumoniae*

There were early indications that antibiotics had a limited lifespan against *S. pneumoniae*. Optochin, which is currently used in the identification of pneumococci, was once in widespread use for the treatment of pneumococcal infections (Moore *et al*, 1917). It was abandoned from clinical therapy because of toxicity and resistance in clinical isolates. Laboratory mediated penicillin resistance was reported in the year prior to the first successful use of the drug for pneumococcal pneumonia. However, it was not until the 1960s that the first penicillin resistant clinical isolate of *S. pneumoniae* was identified (Kislak *et al*, 1965). Despite these early indications of resistance development, penicillin has remained the preferred choice of drug and continues to be used in cases of penicillin sensitive pneumococcal infections. The last few years have seen a rapid increase in the number of antibacterial agents used in the treatment of pneumococcal pneumoniae. This massive effort is due to the high incidence of pneumococcal infections worldwide

and, secondly, the lack of therapeutic agents unaffected by the current resistance mechanisms in the pneumococci (Harwell & Brown, 2000).

Current surveillance data from 1992 to 1996 for community acquired pneumonia shows an increasing trend in antibiotic resistance to the commonly used antibiotic agents. Data confirmed that the clinical utility of penicillin, macrolides and other classes of antimicrobials were seriously compromised by the increasing prevalence of resistance in recent clinical isolates of *S. pneumoniae* to these compounds worldwide (Felmingham & Washington, 1999). The high rates of penicillin resistance found in Spain in the early 1990s, where more than 50% of clinical pneumococci were resistant to one or more antibiotics are now apparent in France, China and the USA (Felmingham & Washington, 1999). Penicillin resistant pneumococci have also been found to harbour diminished susceptibility to other agents like the aminopenicillins, cephalosporins and carbapenams (Felmingham & Washington, 1999). This situation has arisen because of the common targets within the bacterium that are shared by all three classes of agents. Macrolide resistance, particularly in France, Spain, Italy and China, is increasing both in association with and independently of penicillin resistance. In the UK alone, erythromycin resistance has risen from 2.8% to 8.6% between 1990 to 1995 (Felmingham & Washington, 1999). The prevalence of fluoroquinolone resistance in *S. pneumoniae* has only been studied recently. In Canada, it has been shown that fluoroquinolone resistance increased from 0% in 1993 to 1.7% in 1998 (Chen *et al*, 1999). An increase in fluoroquinolone resistance was also reported in South Korea (15%) and Hong Kong (12%) where the incidence of pneumococcal strains with multiple resistance is high (Lee *et al*, 1995; Ho *et al*, 1999). In the UK, the surveillance data published to date

indicates that pneumococcal resistance to the fluoroquinolones is still low (Felmingham & Washington, 1999; Linares *et al*, 1999).

1.7.2 *Haemophilus Influenzae* and *Moraxella Catarrhalis*

Both *H. influ*enzae and *M. catarrhalis* are respiratory tract commensals, which co-reside in the nasopharynx. The entry of these bacteria into the lower respiratory tract combined with an immunocompromised host can result in severe lower respiratory tract infections (Finch, 1995). *Haemophilus influenzae* is a major cause of AECB and acute bacterial rhinosinusitis. The presence of *M. catarrhalis* has also been demonstrated in these infections, albeit at a lower proportion. The mechanism, by which *H. influenzae* and *M. catarrhalis* cause disease is not entirely clear, however, recent work has revealed that it is similar to that of the pneumococcus (Sethi, 2000; Karalus & Campagnari, 2000).

1.7.2.1 Antibiotic Resistance in *H. influenzae* and *M. catarrhalis*

Infections caused by both *H. influenzae* and *M. catarrhalis* are treated primarily by ß-lactams. However, ß-lactamase enzymes are widespread in both species rendering ß-lactam agents like amoxycillin useless (Schito *et al*, 1997). ß-lactam inhibitor combinations like amoxiclav have been used with success although inhibitor resistant TEM (IRT) have been isolated in some strains of *H. influenzae* (Marchese & Schito, 2000). The proportion of ß-lactamase producing strains of *H. influenzae* have risen worldwide as observed in China 38%, USA, Spain, France and Belgium 20–30%, UK and Czech Republic 14.2%, 13% and in the Netherlands and Germany >10% (Felmingham & Washington, 1999). There has also been an emergence of ß-

lactamase negative, ampicillin-resistant strains, which occur only rarely. Azithromycin resistance is rare in *H. influenzae*, although reports of clarithromycin and erythromycin resistance are increasing (Schito *et al*, 1997; Felmingham & Washington, 1999). The only mechanism of resistance of any clinical relevance in *M. catarrhalis* is ß-lactamase production, with more than 90% of isolates doing so (Schito *et al*, 1997), which implies that all amino-penicillins and penicillins are ineffective against *M. catarrhalis*. The use of the fluoroquinolones has been rather limited with these bacteria and since the activity of these agents is rapid and extremely potent, resistance has not arisen as prolifically as seen with the other classes of antibacterials. Both *H. influenzae* and *M. catarrhalis* are exquisitely sensitive to ciprofloxacin, an older generation quinolone with excellent activity against gram-negative bacteria. Surveillance data from the Alexander Project shows that out of 11, 539 clinical isolates of *H. influenzae* tested only 12 strains were found to be resistant and out of 2998 clinical isolates of *M. catarrhalis* only 1 strain was classed as resistant to fluoroquinolones. This is indeed the case, as very few reports have outlined resistance to fluoroquinolones with either species (Felmingham & Washington, 1999).

1.8 Aims of this Thesis

1. To investigate the fluoroquinolone sensitivities in clinical isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* from centres around the United Kingdom.

2. To investigate the mechanisms of fluoroquinolone resistance in laboratory generated mutants of *S. pneumoniae* to various quinolones and the contribution of these mechanisms to resistance to other fluoroquinolone agents.

3. To investigate the mechanisms of fluoroquinolone resistance in clinical isolates of *S. pneumoniae*.

2 Materials and Methods

2.1 Bacterial Strains

Nine hundred and nine clinical isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (See Appendix I) were sent from 9 clinical centres around the United Kingdom and Ireland. Isolate distribution from the various centres is shown in the Figure 2.1 below. The standard bacterial strains used for experiments within this thesis are shown in Table 2.3. All strains were stored at −70°C in cryovials (Alpha Laboratories, Eastleigh, Herts).

Other bacterial isolates used in the execution of the experiments for this thesis are shown in Table 2.4. Verification of individual species was done by rapid diagnostic tests described in Section 2.2 .

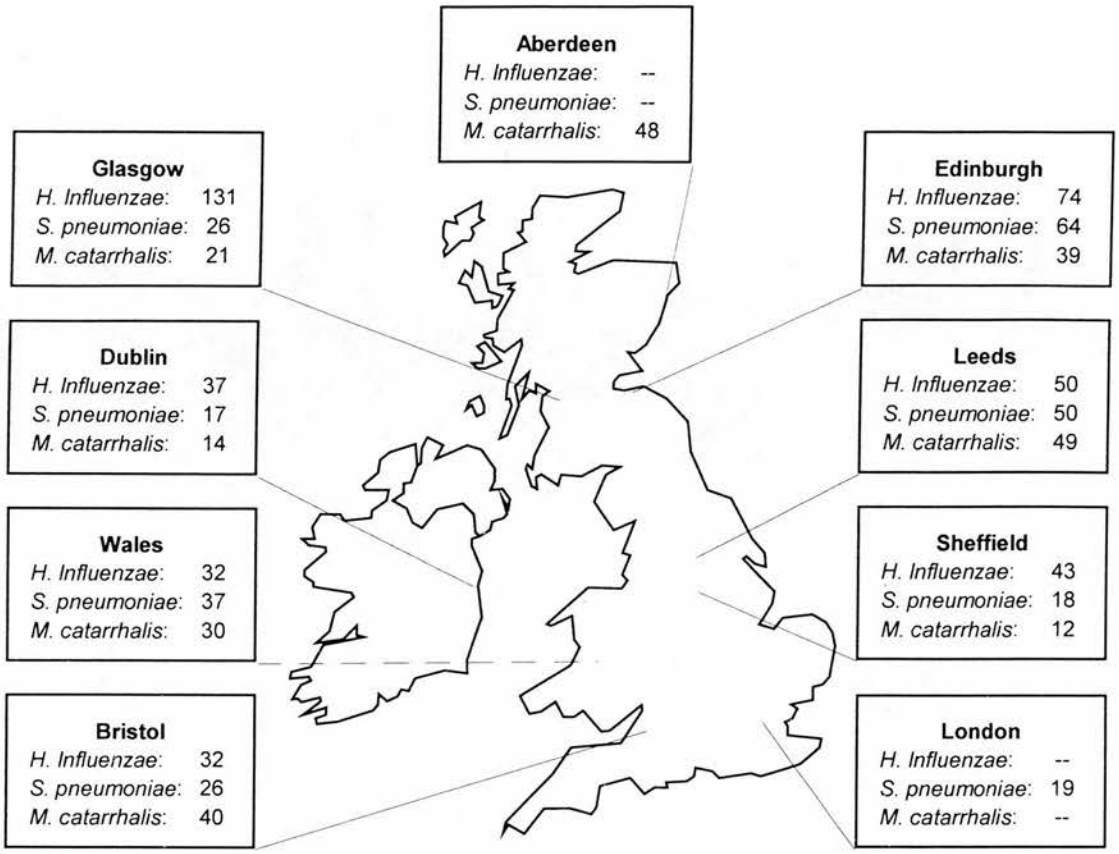


Figure 2.1: Origin of Strains

Numerical figures indicate the number of isolates obtained from respective centres.

The required strains were each inoculated into Todd Hewitt Broth and incubated statically overnight at 37°C in 5% CO_2. Nine hundred microlitres of the overnight broth culture was thoroughly mixed with 100µl of sterile glycerol at a final concentration of 10% $^{v}/v$ and immediately frozen at -70°C. Strains were subcultured directly from the stock to solid media, for verification of phenotype and purity prior to use. Mutant clones obtained from mutation studies were subcultured onto non-selective media (Columbia agar base supplemented with 5% defibrinated horse blood) prior to cryo storage.

Laboratory Standards	Source
Ps. aeruginosa NCTC 10662	National Collection of Type Cultures, London
E. coli 10418	
E. coli β-lactamase positive	
K. pneumoniae NCTC 8773	
S. aureus NCTC 6571	
Methicillin Resistant *S. aureus* S113	
E. faecalis NCTC 51299	
E. faecalis NCTC 12697	
H. influenzae	
H. influenzae β-lactamase positive	
M. catarrhalis	
M. catarrhalis β-lactamase positive	
S. pneumoniae NCTC 7465	
S. pneumoniae 126	
S. pneumoniae 11080	
S. pneumoniae NCTC 13593	
S. pneumoniae R6	Penicillin susceptible, non-capsulated strain from Dr P.V. Adrian, Department of Medical Microbiolgy, University of Witwatersrand, Johannesburg, South Africa.
S. pneumoniae P1Z1/IN27	*S. pneumoniae* R6 transformed with DNA from spontaneous norfloxacin mutant from Dr. N. P. Brenwald, University of Birmingham, Birmingham, UK

Table 2.3: Laboratory Standards Used in Experiments.

Clinical Isolates	Source
7 Penicillin resistant pneumococci collected from centres around the UK	Dr. A. Bamarouf culture collection, University of Edinburgh, Edinburgh, Scotland
13 ciprofloxacin intermediate strains collected from the Royal Infirmary of Edinburgh	Miss F. Walsh culture collection, University of Edinburgh, Edinburgh, Scotland

Table 2.4: Clinical Pneumococcal Isolates Used in Resistance Study

2.2 Identification of Bacterial Isolates

2.2.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae isolates were subcultured onto Columbia agar plates supplemented with 5% defibrinated horse blood. The optochin sensitivity test was performed according to Bowers and Jefferies (1955), distinguishing the pneumococci from viridans streptococci. Optochin discs (Mast Diagnostics, UK) were placed on the confluent region of the subculture prior to incubation at 37°C in 5–10% CO_2. The identity of the pneumococcus was determined when growth inhibition was observed at least 5mm from the margin of the optochin disc.

2.2.2 *Haemophilus influenzae*

Haemophilus influenzae were flood seeded onto a nutrient agar plate (Oxoid, UK) deficient in X and V factors (Mast Diagnostics, UK). One half of the plate was overlaid with discs impregnated with X and V factors separately and XV factors in combination. If growth was only observed around the X and V combination disc, then a positive identification of *H. influenzae* was assumed (Kilian, 1974).

2.2.3 *Moraxella catarrhalis*

A modification of the butyrate hydrolysis strip test (Dealler *et al*, 1989) was used in the identification of *M. catarrhalis*. Colonies of *M. catarrhalis* were emulsified in sterile water and a swab soaked in this emulsion was dipped into a solution of tributyrin tablets (MAST Diagnostics, UK) dissolved in water. If a colour change of

yellow to pink was observed, then a positive identification of *M. catarrhalis* was deduced.

2.3 Growth Media

All growth media and components were sterilised by autoclaving for 15 minutes at 121°C and cooled prior to bacterial inoculation.

2.3.1 Media

All media powders were obtained from Oxoid (Basingstoke, UK) and made up as single strength solutions with distilled water according to the manufacturer's instructions. The media used in these experiments were Brain Heart Infusion (BHI) broth, Todd Hewitt (TH) broth, Columbia agar base.

2.3.1.1 Blood Agar

S. pneumoniae and *M. catarrhalis* strains were subcultured onto blood agar plates. Columbia agar was prepared as described before prior to the addition of 5% defibrinated horse blood and incubated in 5% CO_2 conditions overnight.

2.3.1.2 Chocolate Blood Agar

Haemophilus influenzae strains were grown on lysed blood plates due to the nutritional requirement of the X and V factors. After the addition of 5% defibrinated horse blood to Columbia agar the agar blood mixture was reheated until the whole red blood cells were lysed.

2.4 Chemical Reagents

All reagents used were supplied by Sigma chemicals (Dorset, UK) unless otherwise stated.

2.5 Antibacterial Susceptibility Testing

2.5.1 Antimicrobial Agents

The antimicrobial agents were supplied by various manufacturers found in Table 2.5. Solutions of antimicrobial agents were freshly prepared prior to use in the diluents shown in the table below.

2.5.2 Minimum Inhibitory Concentrations (MICs)

Minimum inhibitory concentrations were performed on Columbia agar base supplemented with 5% defibrinated horse blood following the recommendations of the British Society for Antimicrobial Chemotherapy guidelines (Phillips *et al*, 1991) for susceptibility testing. MICs were determined by the doubling agar dilution method. Agar containing the appropriate concentrations of antimicrobial agents was inoculated with a 2µl spot of test organisms dispensed from a multipoint inoculator (Denley, Billinghurst, Surrey). Bacterial strains to be tested were inoculated into 4.5ml of Todd Hewitt broth or Brain Heart Infusion and incubated at 37°C in 5% CO_2 atmosphere overnight.

Generic Name	Diluent	Abbreviation Used in Text	Supplier
Amoxycillin	Sterile distilled water	AMOX	SmithKline Beecham, UK
Cefotaxime	Sterile distilled water	CTX	Hoechst Marion Roussel, France
Ciprofloxacin	Sterile distilled water	CIP	Bayer AG, Germany
Clarithromycin	Sterile distilled water	CLAR	Abbott Laboratories, UK
Clavulanic Acid	Sterile distilled water	CLAV	SmithKline Beecham, UK
Gatifloxacin	Sterile distilled water	GATI	Grünethal, Germany
Gemifloxacin	Sterile distilled water	GEMI	SmithKline Beecham, UK
Grepafloxacin	Sterile distilled water	GREPA	Glaxo Wellcome, UK
Levofloxacin	Sterile distilled water	LEVO	Hoechst Marion Roussel, France
Moxifloxacin	Sterile distilled water + 1M NaOH	MOX	Bayer AG, Germany
Norfloxacin	Sterile distilled water + 1M NaOH	NOR	Sigma, UK
Penicillin	Sterile distilled water	PEN	Britannia Pharmaceuticals, UK
Sparfloxacin	Sterile distilled water + 1M NaOH	SPAR	Rhone-Poulenc Rorer, France
Tetracycline	Sterile distilled water	TET	Sigma, UK
Trovafloxacin	Sterile distilled water	TROVA	Pfizer, UK

Table 2.5: Antimicrobial Agents and Their Solvents

Turbidity of *S. pneumoniae* cultures was determined to be at 0.5 McFarland standard equivalent to 10^7 cfu prior to inoculation onto antibiotic containing plates. Cultures of *H. influenzae* and *M. catarrhalis* were diluted to 10^4 cfu in physiological saline

before use. Inoculated petri dishes were incubated overnight at 37°C in ambient air conditions in stacks of no more than 3 petri dishes. NCTC standard strains of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were used to validate the experiment.

2.5.2.1 Reserpine Inhibition

Reserpine, a plant alkaloid and efflux pump inhibitor, was added at a concentration of 10mg/l, to the plates with appropriate antibiotic dilutions. A control plate with no antibiotic was also set up to ensure unimpaired growth of isolates in the presence of reserpine. The strain *S. pneumoniae* P1Z1/IN27 which exhibits four-fold reduction in the presence of reserpine to norfloxacin was used as a control (Brenwald *et al*, 1997).

2.5.3 Epsilon Test (E-Test)

The E-test (AB Biodisk, Cambridge, UK) employs a more stringent and convenient method of determining the susceptibilities of bacterial isolates. It comprises a plastic strip embedded with an antibiotic gradient. Overnight broth cultures were used to flood-seed Columbia agar plates supplemented with 5% defibrinated blood. The plastic strips were impregnated on the surface of the agar plates. The plates were then incubated overnight at 37°C in ambient air or in 5% CO_2 in stacks of no more than 3 petri dishes. The manufacturer's instructions were followed in the interpretation of the results.

2.6 Viable Counts

Bacterial strains were grown overnight in 5ml of TH broth by static incubation at 37°C in 5–10% CO_2. Serial dilutions of 1 in 10 and 1 in 100 were made in sterile physiological saline. Subsequently, 0.1ml from each dilution was taken and spread onto Columbia agar base plates supplemented with 5% defibrinated horse blood. The plates were then incubated at 37°C in 5–10% CO_2 overnight. The number of colony forming units per plate were determined with a colony counter (Anderman, Kingston-upon-Thames, UK) and the viable counts within the undiluted cultures were calculated by taking the dilution factor into consideration.

2.7 Mutation Studies

2.7.1 Selecting Quinolone-Resistant Mutants

Quinolone resistant mutants were selected according to the method outlined by Janoir *et al* (1996). Mutant clones were obtained by exposing parent strains of *S. pneumoniae* to stepwise multiples of the MIC to the appropriate quinolone. Exposure to each drug was achieved by plating 100µl of log phase culture onto antibiotic containing plates and incubated for 72 hours at 37°C in 5% CO_2 conditions. Individual clones were selected and subcultured onto non-selective blood agar plates prior to further manipulations. Subsequent generations were raised by challenging of selected clones from each generation. Viable counts were performed simultaneously with mutant selection to allow mutation frequencies to be determined.

2.8 Isolation of Chromosomal DNA

S. pneumoniae isolates were subcultured on Columbia agar supplemented with 5% defibrinated horse blood and incubated in 5% CO_2 at 37°C overnight. Individual colonies were emulsified in sterile milliQ water and boiled for 10 minutes. Subsequently, the crude lysate was used as template for polymerase chain reaction analysis.

2.9 DNA Amplification by Polymerase Chain Reaction

Amplification of the quinolone resistance determining region (QRDR) was performed by PCR. The amplification parameters of the QRDR were as described by Pan & Fisher (1996a).

Component	Volume	Final Concentration	Stock Concentration	Supplier
10 x Taq Buffer	10µl	—	—	Promega, UK
25mM $MgCl_2$	3µl	50mM	2.5mM	Promega, UK
4mM dntp solution[††]	2µl	200µM	4mM	ABI Gene Technologies, UK
Primer A [†] (3′ – 5′)	1µl	10pmol	10pmol/µl	Oswel Technologies
Primer B [†] (5′ – 3′)	1µl	10pmol	10pmol/µl	Oswel Technologies
MilliQ H_2O	to 100µl deionised water	—	—	—
Chromosomal DNA	10µl	—	—	—
Taq Polymerase	2	2 Units	1U/µl	Promega, UK

Table 2.6: Components Used in PCR Reaction.

[††] 4mM dntp stock solution:
1mM dATP, 1mM dCTP, 1mM dGTP, 1mM dTTP in 10mM Tris-Cl (pH 7.5)
[†] For different primers sets used see Table 2.7

Each PCR reaction was performed in a total volume of 100µl (Table 2.6) prepared in a 0.5ml polypropylene microcentrifuge tube (Alpha Laboratories, UK) with Cyclogene thermocycler (Techne, Cambridge, UK) with a heated lid, thereby negating the need for a sterile oil overlay. Each reaction mixture was subjected to 25 or 30 cycles, with each of the three steps: denaturation, annealing and extension. Different cycling protocols were used to amplify the different topoisomerases: *gyrA*, *gyrB*, *parC*, *parE* and the streptococcal efflux pump (PmrA). Primers used for each of the PCR experiments are shown in Table 2.7.

Gene	Primer Sequence	Sequence Reference
DNA Gyrase (Subunit A)	5' CCGTCGCATTCTCTACG 3' 3' AGTTGCTCCATTAACCA 5'	Pan & Fisher, 1996a
Genbank Accession No.	AF053121	
Topoisomerase IV (Subunit C)	5' TGGGTTGAAGCCGGTTCA 3' 3' TGCTGGCAAGACCGTTGG 5'	Pan & Fisher, 1996a
Genbank Accession No.	Z67739	
DNA Gyrase (Subunit B)	5' TGGGCTCCATCGACATCGGC 3' 3' TTGCCAAACGTATCGTAGA 5'	Pan & Fisher, 1996b
Genbank Accession No.	Z67740	
Topoisomerase IV (Subunit E)	5' CCAATCTAAGAATCCTG 3' 3' GCAATATAGACATGACC 5'	Pan & Fisher, 1996b
Genbank Accession No.	Z67739	
PmrA[†]	5' TCAGAACCAGCTTCTTA 3' 3' CTTTTAATAATGTTCGAAA 5'	Gill *et al*, 1999
Genbank Accession No.	AJ007367	

Table 2.7: Primer Oligonucleotide Sequences Used in PCR.

[†] Primers designed with Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer)

The cycling parameters for the different genes are shown in Table 2.8 and Table 2.9 respectively. To validate the reaction, negative and positive controls were incorporated. All primers were synthesised and HPLC purified by Oswel DNA (Southampton, UK) services. All expected PCR product sizes are detailed in Table 2.10.

Segment	Temperature	Time	Cycles
1	95.0	5mins	1
2a	94.0	1min	25
2b	55.0	2mins	
2c	72.0	3mins	
3	72.0	7mins	1

Table 2.8: PCR Heating Cycle Protocol for Topoisomerase Genes (*gyrA*, *gyrB*, *parC* and *parE*)

Segment	Temperature	Time	Cycles
1	94.0	5mins	1
2a	94.0	30s	30
2b	55.0	30s	
2c	72.0	30s	
3	4.0	24hrs	—

Table 2.9: PCR Heating Cycle Protocol for Streptococcal Efflux Pump Gene (*pmrA*)

PCR Reaction	Expected Product Size
DNA Gyrase (Subunit A)	382bp
DNA Gyrase (Subunit B)	444bp
Topoisomerase IV (Subunit C)	366bp
Topoisomerase IV (Subunit E)	357bp
Streptococcal Efflux Pump (PmrA)	1200bp

Table 2.10: Expected Product Sizes for PCR

2.10 Analysis of PCR Products

PCR products were separated at neutral pH values in 2% w/v agarose (GIBCO BRL, Life Technologies, UK) gels in TAE buffer [40mM Tris-acetate pH7.6, 1mM EDTA]. Under these conditions, double stranded DNA is negatively charged hence

loaded near the cathode and migrates towards the anode with the application of an electric field (Aaij & Borst, 1972). Gel electrophoresis was carried out in a minisubcell GT (Biorad, UK) under the constant voltage of 100V (Powerpac 300, Biorad, UK) for 40 minutes, depending on the migration of the bands. For larger gels subcell GT (Biorad, UK) gel tanks were used. A DNA ladder (Generuler^{TM}, MBI, Fermentas) was run alongside the PCR products for the verification of the sample's molecular weight. Each DNA sample was mixed with 2μl of loading buffer [30% w/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol] before loading the wells. The electrophoresed fragments were visualised after electrophoresis by staining for one hour in a 50μg/ml ethidium bromide solution and viewed on an UV transilluminator (UV products, Cambridge, UK).

2.11 Purification of DNA

PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. The final volume for elution of the purified products was reduced to 30μl to concentrate the DNA. The DNA concentration of the PCR product was approximated by comparing the intensity of bands after electrophoresis of λ DNA digested with *Hind*III (Sigma, UK) known to contain 0.5μg per microlitre.

2.12 Automated DNA Sequencing

PCR primers were also used as sequencing primers at a concentration of 3.2pmol per reaction. Purified DNA was processed as described in Section 2.11 prior to

sequencing to ensure that the required concentration of 30–90ng was achieved. The DNA sequence was determined by the chain termination method developed by Sanger *et al* (1977). Individual PCR fragments were set up in the Ready Reaction Format for fluorescence based on dideoxy cycle sequencing (PE Applied Systems, UK). All sequences were processed in the Department of Haematology, Royal Infirmary of Edinburgh. Sequences were analysed by the BLAST online search engine (http://www.ncbi.nih.gov/cgi-bin/BLAST) with the quinolone susceptible parent strain sequence in the database. Comparisons between the sequences of the mutant clones of clinical strains were determined by comparing the parent strains and selected mutant progeny from each generation using the multialin interface website (http://www.toulouse.inra.fr/multalin.html).

2.13 Probe Construction

The PCR DIG Probe synthesis kit (Roche Molecular Biochemicals, Germany) was used to manufacture the 1.2kb DIG labelled probe for the streptococcal efflux pump *pmrA*. The primers used in this PCR were synthesised by Oswel DNA service (Southampton) designed with Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer) based on the published gene sequence. Each PCR reaction was performed in a total volume of 50µl. A positive control consisting of the PCR reagents and template DNA from P1Z1/IN27, an efflux mutant (Gill *et al*, 1999) was used. The negative control consisted of PCR reagents without any template DNA. The cycling parameters and the reagent volumes for the labelling PCR are shown in Table 2.9 and Table 2.11 respectively.

The PCR products were verified by agarose gel electrophoresis as detailed in Section 2.10 .

Reagents	Volume	Final Concentration
Sterile Milli Q water	27.25µl	Variable
PCR Buffer with $MgCl_2$ (10 X)	5µl	1 X
Dntps(1:1)	5µl	200µM
Primer A	1µl	0.1 — 1µM
Primer B	1µl	0.1 — 1µM
Enzyme mix	0.75µl	2.6Units
Template DNA	10µl	Variable
Total	50µl	—

Table 2.11: Reagents Used to Synthesise Probes

2.14 Restriction Fragment Length Polymorphism (RFLP)

2.14.1 Restriction of Products from Topoisomerase PCR

RFLP was performed to distinguish between the sensitive and resistant *gyrA* and *parC* by the loss of a single nucleotide, resulting in an amino acid change from Serine81 → Leucine/Phenyalanine or Serine79 → Tyrosine/Phenylalanine respectively (Varon & Gutmann, 2000). This substitution manifests itself through the loss of the *Hinf*1 recognition site of G' ANTC. Subsequently, PCR products of *gyrA* and *parC* QRDR were restricted using *Hinf*1 (Promega, UK). The restriction digest comprised of 1µl of *Hinf*1 enzyme (10U/µl), 5µl of restriction buffer [10mM Tris-Cl, pH7.5, 60mM $NaCl_2$, 7mM $MgCl_2$], 1µl of BSA (0.1mg/l), 4µl of milliQ water and 40µl of amplified PCR product. Digestion was carried out for a maximum of two

hours at 37°C. The resulting amplimers were electrophoresed on a 2% agarose gel and visualised.

2.14.2 Restriction of Products from Streptococcal Efflux Pump PCR

To ensure the validity of the streptococcal efflux pump PCR fragments generated by both the DIG probe synthesis and normal PCR, restriction digests with *Cla*1 (Promega, UK) was done. The conditions of the digest are as described for the *Hinf*1 digest.

2.15 Accumulation Assays

Accumulation assays were performed according to the procedure detailed by Mortimer & Piddock (1991). Briefly, 40µl of overnight broth culture of the test organism was inoculated into 10ml of pre-warmed BHI broth and incubated shaking at 37°C until an optical density of 0.7–0.8units was reached at 660nm. Cells were harvested by centrifugation in the Sorvall RT 6000D (Dupont, UK) at 4°C (3000rpm, 20 minutes). The resulting cell pellets were washed by resuspending in 10ml of cold 50mM phosphate buffer (pH7.0). The mixture was recentrifuged and resuspended to yield an optical density of 20 units at OD_{660}. The suspension was then transferred to a sterile McCartney bottle, placed in a static waterbath at 37°C and allowed to equilibrate. Cell suspensions were plated to ensure purity and identity of the test organism prior to further manipulations. At the initial time point (t=0), 500µl of cell suspension was removed and placed on ice. Moxifloxacin were added to a final concentration of 10µg/ml. Duplicate 500µl samples were removed at time intervals

of 10s up to 5 minutes. Samples were centrifuged in the Sorvall RT 6000D (Dupont, UK) at 4°C (3000 rpm, 5 minutes) and returned to ice. The supernatant was discarded and the cell pellet was resuspended in 1ml of sterile phosphate buffer (50mM sodium phosphate buffer, pH7.0) to remove the cell bound quinolone. The cell suspensions were recentrifuged and resuspended in 1ml of 0.1M glycine (pH3) and left at room temperature for 2 hours. Resulting samples were centrifuged at 13,000rpm (11600×g) in the MSE micocentaur centrifuge for 5 minutes then transferred to a fresh eppendorf and recentrifuged under the same conditions. Fluorescence was measured with a F2000 fluorescence spectrophotometer (Hitachi, UK) at the appropriate excitation and emission wavelengths detailed in Table 2.12. The uptake of moxifloxacin from the cells was measured as an increase in fluorescence. Graphs of fluorescence versus time (minutes) were plotted.

Drug	Excitation Wavelength (nm)	Emission Wavelength (nm)
Moxifloxacin	294	504

Table 2.12: Excitation and Emission Wavelength of Moxifloxacin
(Piddock & Jin, 1999)

2.16 Ribonucleic Acid (RNA) Analysis

2.16.1 RNA Extraction

Total RNA was extracted with the SV Total RNA Isolation System (Promega, UK) from bacterial cultures grown to an optical density of 0.6–1.0 at OD_{600} (Kobs, 1998). Contaminating RNAases were eradicated from the benches and equipment by 3% (v/v) hydrogen peroxide and RNAZAP (Ambion, UK).

2.16.2 RNA Induction Assays

To determine whether the streptococcal efflux pump, *pmrA* could be induced by both moxifloxacin and ciprofloxacin, induction experiments were done. Overnight broth cultures of *S. pneumoniae* R6, *S. pneumoniae* P1Z1/IN27 and *S. pneumoniae* 285 were subcultured to three hour logarthmic phase cultures by adding 1ml of overnight broth culture to 9ml of prewarmed TH broth. All log phase cultures were simultaneously induced with the concentration of antibiotic equivalent to half the MIC for both quinolones (Table 2.13). Control broth cultures with no antibiotic were also set up. The RNA was then extracted from all broth cultures of the strains with the SV Total RNA Isolation System (Promega, UK).

Strain	Concentration (mg/l)	
	Ciprofloxacin	**Moxifloxacin**
S. pneumoniae R6	0.25	0.0312
S. pneumoniae P1Z1/IN27	1	0.0312
S. pneumoniae 285	0.5	0.0312

Table 2.13: Quinolone Concentrations Used in the Induction Experiment

2.16.3 RNA Dot Blots

The RNA dot blots were prepared by spotting 10µl of extracted total RNA samples onto nylon membranes (Amersham Pharmacia Plc, UK). The membranes were subsequently dried and baked at 80°C for two hours prior to hybridisation and exposure.

2.16.4 Electrophoresis of RNA

RNA was electrophoresed on a 1% formaldehyde based agarose gel. The gel was prepared by melting 1g of agarose in 1 × MOPs buffer [200mM morphinopropane sulfonic acid, 50mM sodium acetate and 10mM EDTA, pH7.0]. After cooling, 5.4ml of 37% (v/v) formaldehyde was added and thoroughly mixed before casting in the gel mould. Ten microliter volumes of the frozen RNA samples were vacuum dried with the freeze dryer (Modulyo 4L, Cryotechnics, UK) prior to further manipulations. The formaldehyde gel loading buffer mixture comprised of 720µl formamide, 160µl of 10 × MOPs Buffer, 260µl of 37% (v/v) formaldehyde, 100µl of DEPC-treated sterile MilliQ water, 100µl of ethidium bromide (10mg/ml), 80µl of sterile glycerol and 80µl of saturated bromophenol blue in water. Twenty microliters of the gel loading buffer were added to the freeze-dried samples, mixed and boiled for two minutes prior to loading onto the gel. The gel was electrophoresed for 2–3 hours at a 100V in 1× MOPs buffer. Due to the electropotential of ethidium bromide and bromophenol blue, migration towards the cathode (-ve) and anode (+ve) will occur respectively. To prevent unnecessary background, the topmost section of the gel was excised, before the ethidium bromide exited the gel matrix. The presence of intact RNA was confirmed by visualisation on a UV transilluminator. The gel was then soaked in 2 changes of 10 × SSC buffer [20 × SSC: 3M NaCl, 0.3M Na citrate, pH7] for 20 minutes separately, to ensure the removal of the formaldehyde from the gel and finally immersed in DEPC-treated Milli Q water once prior to transfer.

2.16.5 Northern Blot Transfer

Downward alkaline transfer to the nitrocellulose membranes was done for exactly three hours according to the manufacturer's guidelines (Turboblotter™, Schleicher and Schuell). Following neutralisation and transfer, the RNA was immobilised by baking at 80°C for 2 hours. Blots were then stored at 4°C until use.

2.16.6 Hybridisation of Northern Blots

Hybridisation of northern blot filters was carried out following the protocol detailed by Stratagene™ (1999). The probe used was a fluorescein labelled PCR fragment of the streptococcal efflux pump *pmrA* (as described in Section 2.13) and added at a concentration between 5–25ng/ml (according to manufacturer's recommendations for DIG labelling kit). The hybridisation buffer [5 × SSC, 50% deionized formamide, 2% Blocking agent, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS] was warmed to 42°C with the nylon membrane before the addition of the probe. Approximately, 25ng/ml of probe was boiled for 5 minutes, chilled immediately and added to the remaining hybridisation solution. The blot was hybridised overnight at 42°C in a hybrisation oven (Hybridiser, HB-1D, Techne, UK). The blot was then washed in 0.1% SSC and 0.1% (w/v) SDS thrice, separately for an hour at 68°C each time with gentle shaking.

2.16.7 Detection of Blots

The detection of digoxigenin-labelled nucleic acids was done according to the manufacturer's guidelines (Boehringer Mannheim, Germany). Autoradiography was

carried out by exposure to Hyperfilm X-Ray film (Amersham Pharmacia Plc, UK) at room temperature for 30mins – 1hour, before developing the film in an automatic film processor.

3 Results

3.1 Prologue

The results of the research executed for the completion of this thesis has been divided into six sections. The first section describes the discrepancies observed with the susceptibility testing when performed under different incubation conditions. This illustrates the requirement for a unified testing procedure within different laboratories to allow direct comparisons of susceptibility data and also the influence variable testing conditions have on the subsequent categorisation of strains as sensitive, intermediate or resistant. The second section applies the observations of the first section in the testing procedure of a UK wide susceptibility study involving respiratory isolates namely *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Clinical isolates of *S. pneumoniae* that showed decreased susceptibility to the

fluoroquinolones were further analysed to determine the mechanisms of resistance to these antibacterials.

To investigate the selection pressure exerted by the newer extended-spectrum quinolones on the current clinical pneumococcal population, laboratory mutants were generated from isolates with different pneumococcal phenotypes by moxifloxacin challenge. These data hold crucial significance as they not only track the evolution of resistance mechanisms that a given population experiences when challenged with moxifloxacin and older agents such as ciprofloxacin and norfloxacin but also disputes the hypothesis that topoisomerase IV is the primary target of all fluoroquinolones in *S. pneumoniae*.

Fluoroquinolone resistance has also been attributed to arise through efflux pump mutations. In *S. pneumoniae,* the recently described pump *pmrA* has been shown to play a role in the acquisition of ciprofloxacin and norfloxacin resistance. To determine whether *pmrA* does contribute to the evolution of moxifloxacin resistance, inhibition studies using an efflux pump inhibitor reserpine were conducted with isogenic mutants derived after moxifloxacin challenge and clinical pneumococcal isolates with reduced fluoroquinolone susceptibility. In order to determine moxifloxacin uptake within resistant mutants, accumulation assays using the fluorescence technique were done. Further analysis of the role of the efflux pump was evaluated through northern blot analysis.

3.2 Susceptibility Testing

The assessment of the efficacy of antibacterial agents *in vitro* has been traditionally determined by doubling agar dilutions. The validity of this procedure is governed by several factors i.e. media used, incubation conditions which readily affect the outcome of the sensitivity results. Different incubation conditions are required by different species of bacteria and some of these incubation conditions have been shown to affect the activity of some classes of antibiotics e.g. macrolides (Phillips *et al*, 1991). The BSAC guidelines state that "10% (v/v) carbon dioxide in the atmosphere will reduce the pH of the media surface thereby affecting the growth of the organism or the antibacterial action of some antibiotics e.g. aminoglycosides, macrolides and quinolones". The acidification of media by the decrease in pH has been shown to directly affect the antibacterial activity of antibiotics such as the macrolides and quinolones, which are pH dependent in their *in vitro* activity (Phillips *et al*, 1991).

The recent introduction of anti gram-positive quinolones with extended efficacy towards pathogens which are grown in reduced air conditions requires a reassessment of the testing procedure. A study done by Bruggemann *et al* (1997) demonstrated the reducing effects carbon dioxide had on the testing media and the activity of the antibiotic that was incorporated in it. Carbon dioxide incubation was demonstrated to reduce the pH on the agar surface from 7.40 to 6.98 and subsequently the MICs reported from those plates incubated in the CO_2 rich atmosphere were elevated in comparison to those obtained in air. This prompted the investigators to perform all subsequent sensitivity testing in air. Therefore, to verify this and elucidate the effect

of incubation in oxygen enriched and oxygen-reduced atmosphere (5% carbon dioxide), the MICs of 18 NCTC standard strains were determined under both conditions.

The MICs were determined, by the doubling agar dilution method and the Epsilon test (E-Test), for the following extended-spectrum fluoroquinolones trovafloxacin, moxifloxacin, grepafloxacin, and an older comparator ciprofloxacin. Two sets of MIC plates were inoculated with the multipoint inoculator. All isolates were also inoculated onto antibiotic-free plates to confirm growth under both incubation conditions. The plates were read after an 18 hour incubation period, where, the lowest concentration of antibiotic that inhibited growth was defined as the MIC and single colonies or hazy growth were ignored. The MIC was expressed as milligrams per litre and the increases in the MICs were recorded for the carbon dioxide incubations and compared against oxygen.

3.2.1 Doubling Agar Dilution Results in Oxygen and Carbon Dioxide Incubation.

Against *S. pneumoniae*, 2–4 fold increases in the MIC were observed with all the quinolones tested under the reduced air conditions (5% carbon dioxide) (See Table 3.1). With *S. pneumoniae* NCTC 13593 and NCTC 126 the four-fold increase in moxifloxacin MIC recorded under reduced air conditions would have resulted in the categorisation of this strain as resistant to moxifloxacin based on the recommended breakpoint value (Andrews *et al*, 1999). Similarly, for *S. pneumoniae* R6 a four-fold increase was observed with ciprofloxacin from 0.25mg/l to 1mg/l, resulting in the classification of this strain as being intermediately resistant to

ciprofloxacin. Interestingly, the trovafloxacin MIC was 2–4-fold lower when incubated in carbon dioxide for both *S. pneumoniae* R6 and *S. pneumoniae* NCTC 7465 although, an 8-fold increase in the trovafloxacin MIC was observed with both *S. pneumoniae* 126 and 11050.

Strain	Moxi-floxacin (O_2)	Moxi-floxacin (CO_2)	Trova-floxacin (O_2)	Trova-floxacin (CO_2)	Cipro-floxacin (O_2)	Cipro-floxacin (CO_2)
Spn R6	0.0312	0.0625	0.125	0.0625	0.25	1
Spn 7465	0.0312	0.0625	0.5	0.0625	0.25	0.25
Spn 13593	0.25	1	0.5	0.5	16	64
Spn 126	1	4	0.5	4	16	64
Spn 11050	0.125	0.5	<0.0039	0.5	8	8
Hi	<0.0039	0.0312	0.0078	0.0312	<0.0039	0.0625
Hi ß+ve	0.0312	0.125	0.0312	0.25	0.0078	0.0312
M.cat	0.0312	0.125	0.0312	0.0312	0.125	0.25
M.cat ß+ve	0.0312	0.0625	0.0078	0.0312	0.0312	0.0625
E.coli	0.0156	0.0312	0.0078	0.0312	0.0156	0.0312
E.coli ß+ve	0.0312	0.0625	0.0156	0.0312	0.0156	0.0312
Kpn 8773	0.0312	0.0625	0.0156	0.0312	0.0156	0.0312
Ps.aero	0.5	2	1	0.5	0.125	0.125
E.faec 51299	0.0625	0.125	0.0312	0.125	0.25	0.5
E.faec 12697	0.0625	0.125	0.0312	0.125	0.25	0.5
S.aer 6571	0.0156	0.0312	0.0156	0.0312	0.0625	0.125
MRSA S113	0.0156	0.0312	0.0078	0.0312	0.5	0.5
EMRSA 15	1	2	2	1	32	32

Table 3.1: Minimum Inhibitory Concentration of Control Strains Done in Oxygen and Carbon Dioxide Incubation

Against ciprofloxacin, a four–fold increase was observed with carbon dioxide incubation except for *S. pneumoniae* 7465 and 11050. Against both *H. influenzae* and *M. catarrhalis* MIC increases of 2–4 fold, were observed with all the

fluoroquinolones when values from the reduced air atmosphere were compared to oxygen incubation. Similarly, against gram-negative pathogens such as *E. coli*, *K. pneumoniae* and *Ps. aeruginosa* 2–4-fold increases in the MICs were observed in the 5% carbon dioxide incubation. The most pronounced increase was seen with *Ps. aeruginosa* and moxifloxacin where a four-fold increase in the MIC was observed in carbon dioxide incubation. This is the highest increase that was recorded for an organism, which does not require reduced air conditions for growth. *E. faecalis* and *S. aureus* showed moderate 2-fold increases to all the fluoroquinolones tested under reduced air conditions (5% carbon dioxide).

3.2.2 Epsilon Test Results in Oxygen and Carbon Dioxide

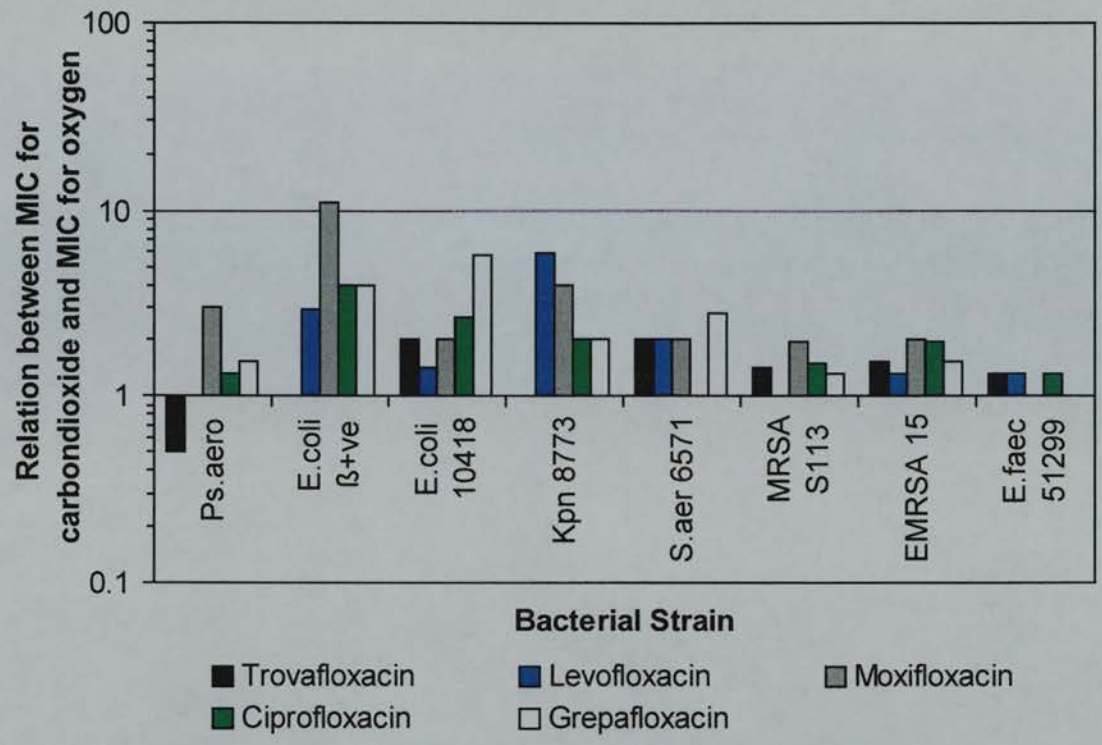
To further elucidate the differences in oxygen and 5% carbon dioxide incubations, MIC determinations were performed with the E-test strips, where the antibiotic is not incorporated into the media. The results obtained with the E-test mirrored those of the doubling agar dilution method. Columbia agar plates supplemented with 5% defibrinated horse blood were flood-seeded with standardised overnight cultures of the organism under test. The results given in Table 3.2 are the results obtained in ambient air, followed by Table 3.3 with the results obtained in carbon dioxide.

Strain	Moxi-floxacin	Trova-floxacin	Grepa-floxacin	Levo-floxacin	Cipro-floxacin
Spn 7465	0.064	0.032	0.016	0.094	0.008
Spn 126	0.25	0.094	0.75	1.5	4
Spn 11080	0.19	0.25	0.38	2	6
Spn 13593	0.25	0.38	0.50	2	8
Hi	0.016	0.008	0.004	0.012	0.012
Hi ß+ve	0.25	0.25	0.125	0.50	0.25
M.cat	0.047	0.047	0.012	0.047	0.023
M.cat ß+ve	0.064	0.016	0.012	0.047	0.047
E.coli 10418	0.008	0.002	0.004	0.016	0.006
E.coli ß+ve	0.023	0.016	0.008	0.032	0.008
Kpn 8773	0.032	0.032	0.032	0.016	0.023
Ps.aero	0.50	0.38	0.125	0.38	0.094
E.faec 51299	0.125	0.125	0.25	0.75	0.38
E.faec 12697	0.125	0.094	0.25	0.75	0.38
S.aer 6571	0.023	0.006	0.023	0.094	0.094
MRSA S113	0.064	0.016	0.094	0.25	0.50

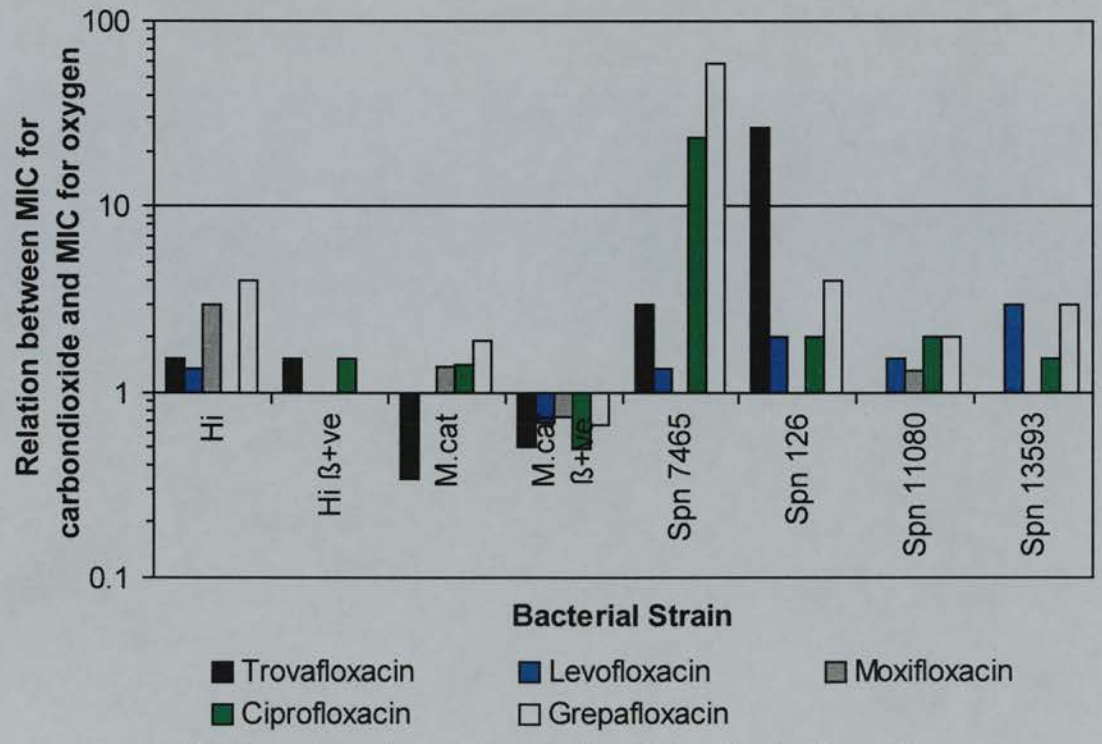
Table 3.2: Epsilon Test Results Obtained with Control Strains when Tested in Oxygen

Strain	Moxi-floxacin	Trova-floxacin	Grepa-floxacin	Levo-floxacin	Cipro-floxacin
Spn 7465	0.064	0.094	0.094	0.125	0.19
Spn 126	0.25	0.25	3	3	8
Spn 11080	0.25	0.25	0.75	3	12
Spn 13593	0.25	0.38	1.5	6	12
Hi	0.047	0.012	0.016	0.016	0.012
Hi ß+ve	0.25	0.38	0.125	0.50	0.38
M.cat	0.064	0.016	0.023	0.047	0.032
M.cat ß+ve	0.047	0.008	0.008	0.032	0.023
E.coli 10418	0.016	0.004	0.023	0.023	0.016
E.coli ß+ve	0.25	0.016	0.032	0.094	0.032
Kpn 8773	0.125	0.032	0.064	0.094	0.047
Ps.aero	1.5	0.19	0.19	0.38	0.125
E.faec 51299	0.25	0.19	0.38	1.0	0.75
E.faec 12697	0.125	0.125	0.25	1.0	0.5
S.aer 6571	0.047	0.012	0.064	0.19	0.094
MRSA S113	0.125	0.023	0.125	0.25	0.75

Table 3.3: Epsilon Test Results Obtained with Control Strains when Tested in Carbon Dioxide



(a) Organisms not Requiring Carbon Dioxide for Growth



(b) Organisms Requiring Carbon Dioxide for Growth

Figure 3.1: Differentials Between Carbon Dioxide and Oxygen Athmosphere

Increases in MICs observed in CO_2 are represented by bars above the horizontal axis, increases in O_2 are represented by bars below the horizontal axis.

With the key respiratory pathogens (*S. pneumoniae, H. influenzae and M. catarrhalis* differentials observed between 5% carbon dioxide and oxygen ranged from 1.3–23.7 times. Some increases were observed with oxygen incubation but these were not as pronounced as those observed in carbon dioxide incubations. A graphical description of this relationship between the MICs is shown in Figure 3.1. Only a 1.3 fold increase in the moxifloxacin MIC was observed with *S. pneumoniae* 11080 in contrast to the results obtained with the doubling agar dilutions. Against enterobacteriaceae and *Ps. aeruginosa*, there were increases observed with all the fluoroquinolones tested. Again, a 3-fold increase in the moxifloxacin MIC was observed with *Ps. aeruginosa* when incubated in 5% carbon dioxide conditions. Thus, it appears that 5% carbon dioxide incubations do have an effect on the antibacterial potency of the fluoroquinolones and is similar to that reported with the macrolides (Johnson *et al*, 1999).

Interestingly, with both incubation conditions, increases were observed with the trovafloxacin MIC values obtained in air in comparison to the 5% carbon dioxide incubation. With the doubling agar dilution method, 2–8 fold increases were observed with the trovafloxacin MIC and *Ps. aeruginosa* and *S. pneumoniae* 7465 when compared with the 5% carbon dioxide incubation MIC.

3.2.3 Concluding Remarks

Consistently, elevated MICs were observed in the control organisms when incubation was performed in a oxygen reduced atmosphere (5% carbon dioxide). The increases were observed with both gram-positive pathogens which are traditionally incubated in carbon dioxide and gram-negative pathogens like *Ps. aeruginosa* and *E. coli*

which are not. Generally, all the quinolones except trovafloxacin tested exhibited increased MICs in 5% carbon dioxide conditions. In both susceptibility testing procedures, it was found that the most pronounced increases were observed with the pneumococcal isolates when tested against grepafloxacin, levofloxacin and ciprofloxacin. Elevated MICs hinder the interpretation of the genuine potency of the compound being tested and also the susceptibility profile of the target clinical population. Therefore, the subsequent survey performed all incubations for MIC determinations in air.

3.3 Moxifloxacin Sensitivity Study

Nine hundred and nine clinical respiratory isolates were collected from nine geographically distinct centres across the United Kingdom (See Figure 2.1). Clinical isolates *of Streptococcus pneumoniae* (n=257), *Haemophilus influenzae* (n=399) and *Moraxella catarrhalis* (n=253) were obtained between March 1998 and April 1999. Identification of the isolates was carried out in the individual centres prior to their arrival in Edinburgh. However, some isolates were reanalysed through the standard biochemical tests and API 20NE tests in Edinburgh to reconfirm the identity before experimental manipulations. The strains were designated with prefixes indicating the species type and strain numbers sequentially. The original site of isolation or any further details regarding patient information of all the clinical isolates was not provided by the participating centers. Susceptibility profiles (MICs) were determined by the doubling agar dilution method to penicillin, amoxycillin and amoxycillin+clavulanic acid, cefotaxime, tetracycline, clarithromycin, moxifloxacin, trovafloxacin, grepafloxacin, sparfloxacin, levofloxacin and ciprofloxacin. With some strains, the values were verified by E-test in addition to the agar dilution method. Strains exhibiting decreased susceptibility to the fluoroquinolones were further investigated to determine the mechanisms of resistance. Initially, the polymerase chain reaction (PCR) was done to amplify the subunit A (*gyrA*) of DNA gyrase and the C subunit (*parC*) of topoisomerase IV. Subsequently the products of this reaction were examined through restriction fragment length polymorphisms and dideoxy sequencing. These results were compared with quinolone susceptible strains within the GenBank database to identify changes within these genes that contribute towards fluoroquinolone resistance.

Organism	Antimicrobial Agent	Range	MIC_{50}	MIC_{90}
Streptococcus pneumoniae (n = 257)	Penicillin	<0.008 – 4	0.0156	1
	Cefotaxime	<0.004 – 2	0.0625	1
	Tetracycline	<0.004 – 64	0.25	32
	Clarithromycin	0.004 – >16	0.0156	1
	Moxifloxacin	0.004 – 0.5	0.0625	0.25
	Trovafloxacin	<0.004 – 1	0.0625	0.5
	Grepafloxacin	<0.004 – 1	0.0625	0.25
	Sparfloxacin	<0.004 – 2	0.0625	0.5
	Levofloxacin	<0.004 – 2	0.5	1
	Ciprofloxacin	<0.004 – 8	0.5	2
Haemophilus influenzae (n = 399)	Amoxycillin	<0.25 – 64	1	64
	Amoxycillin + Clavulanic Acid	<0.25 – 2	0.5	1
	Cefotaxime	<0.004 – 16	0.0312	0.0625
	Tetracycline	<0.004 – 16	1	4
	Clarithromycin	0.016 – >16	4	8
	Moxifloxacin	<0.004 – 0.25	<0.004	0.0078
	Trovafloxacin	<0.004 – 0.5	<0.004	0.0078
	Grepafloxacin	<0.004 – 0.25	<0.004	0.0078
	Sparfloxacin	<0.004 – 0.0078	<0.004	0.0078
	Levofloxacin	<0.004 – 0.25	0.0156	0.0625
	Ciprofloxacin	<0.004 – 1	0.0078	0.0156
Moraxella catarrhalis (n = 253)	Amoxycillin	<0.125 – 32	2	16
	Amoxycillin + Clavulanic Acid	<0.125 – 2	0.25	0.5
	Cefotaxime	0.0078 – 4	0.5	1
	Tetracycline	0.004 – 2	1	2
	Clarithromycin	0.004 – 16	0.0625	0.5
	Moxifloxacin	<0.004 – 0.25	0.0312	0.125
	Trovafloxacin	0.004 – 0.5	0.0312	0.5
	Grepafloxacin	<0.004 – 0.5	<0.004	0.0625
	Sparfloxacin	<0.004 – 0.5	0.0078	0.125
	Levofloxacin	<0.004 – 2	0.0625	0.125
	Ciprofloxacin	<0.004 – 2	0.0312	0.125

Table 3.4: Minimum Inhibitory Concentration of Respiratory Pathogens

All strains were incubated overnight (18–24hrs) prior to inoculation. 18hr aerobic incubation at 37°C following inoculation of columbia agar plates supplemented with 5% whole horse blood (*S. pneumoniae* and *M. catarrhalis*) and lysed blood (*H. influenzae*) containing the appropriate concentration of antibiotic with 2µl of standardised test organism (0.5M McFarland) culture. All experiments were validated by the use of the following controls: *S. pneumoniae* NCTC 7465, *M. catarrhalis*, *H. influenzae* NCTC 11931.

The MIC_{50} and MIC_{90} of 909 clinical respiratory isolates are shown in Table 3.4 with the range of MICs observed for each antibiotic. All three respiratory pathogens exhibit MIC_{50} and MIC_{90} below the recommended breakpoint (1mg/l) (Andrews *et al*, 1999) against moxifloxacin. Against *Streptococcus pneumoniae* moxifloxacin and grepafloxacin were the most potent antibacterials (MIC_{90} = 0.25mg/l) (See Figure 3.5). Trovafloxacin was the next most active (MIC_{90} = 0.5mg/l) followed by levofloxacin and ciprofloxacin. Examining the MIC distribution for *S. pneumoniae* for the fluoroquinolones demonstrated that moxifloxacin had equal or better activity in comparison to the comparator quinolones. Penicillin was as active as cefotaxime and clarithromycin (MIC_{90} = 1mg/l) (See Figure 3.2, Table 3.4) against *S. pneumoniae*. Versus *Haemophilus influenzae* grepafloxacin (MIC_{90} = < 0.004mg/l) was found to be the most active fluoroquinolone. Moxifloxacin and trovafloxacin were the next most active (MIC_{90} = 0.0078mg/l). Clarithromycin was the least active against *Haemophilus influenzae* (MIC_{90} = 8mg/l) (Figure 3.4, Table 3.4). Against *Moraxella catarrhalis,* grepafloxacin inhibited at 0.0625mg/l followed by moxifloxacin, sparfloxacin, levofloxacin and ciprofloxacin. Trovafloxacin, clarithromycin and tetracycline were found to have the least activity at 0.5mg/l (MIC_{90}) (Figure 3.3, Table 3.4). Generally, moxifloxacin was found to be 2–4-fold more active versus the three pathogens when compared to the β-lactams, tetracycline and clarithromycin.

β-lactam resistant *H. influenzae* are increasingly common and to determine the relationship between β-lactam resistance and quinolone resistance, *H. influenzae* isolates that demonstrated amoxycillin MICs ≥ 16mg/l and ≤ 8mg/l were sorted to identify corresponding increases in fluoroquinolone MICs (See Table 3.5).

Organism	Antimicrobial Agent	MIC (mg/l)		
		Range	MIC_{50}	MIC_{90}
H. influenzae	Amoxycillin + Clavulanic Acid	<0.25 – 2	0.5	1
Amox ≥ 16mg/l	Cefotaxime	0.0156 – 16	0.0312	0.0625
(n = 33)	Tetracycline	0.0125 – 16	1	8
	Clarithromycin	0.016 – 16	4	8
	Moxifloxacin	<0.004 – 0.125	0.0078	0.0312
	Grepafloxacin	<0.004 – 0.0078	<0.004	<0.004
	Sparfloxacin	<0.004 – 0.0078	<0.004	0.0078
	Levofloxacin	<0.004 – 0.25	0.0312	0.0625
	Ciprofloxacin	<0.004 – 1	0.0078	0.0156
	Trovafloxacin	<0.004 – 0.5	<0.004	0.0078
H. influenzae	Amoxycillin + Clavulanic Acid	<0.25 – 1	0.5	1
Amox ≤ 8mg/l	Cefotaxime	<0.004 – 2	0.0156	0.0312
(n = 366)	Tetracycline	<0.004 – 16	1	2
	Clarithromycin	0.016 – 16	4	8
	Moxifloxacin	<0.004 – 0.25	<0.004	0.0156
	Grepafloxacin	<0.004 – 0.0156	<0.004	0.0078
	Sparfloxacin	<0.004 – <0.004	<0.004	<0.004
	Levofloxacin	<0.004 – 0.0625	0.0156	0.0625
	Ciprofloxacin	<0.004 – 0.0156	0.0078	0.0156
	Trovafloxacin	<0.004 – 0.0312	<0.004	0.0156

Table 3.5: Minimum Inhibitory Concentration of Amoxycillin Sensitive and Amoxycillin Resistant *H. influenzae*

As shown in Table 3.5, the quinolone MICs remain unchanged or differ by a two fold dilution when compared for *H. influenzae* which show ≤8mg/l or ≥16mg/l to amoxycillin. This result is also represented by penicillin resistant, intermediate and sensitive pneumococci (Data not shown).

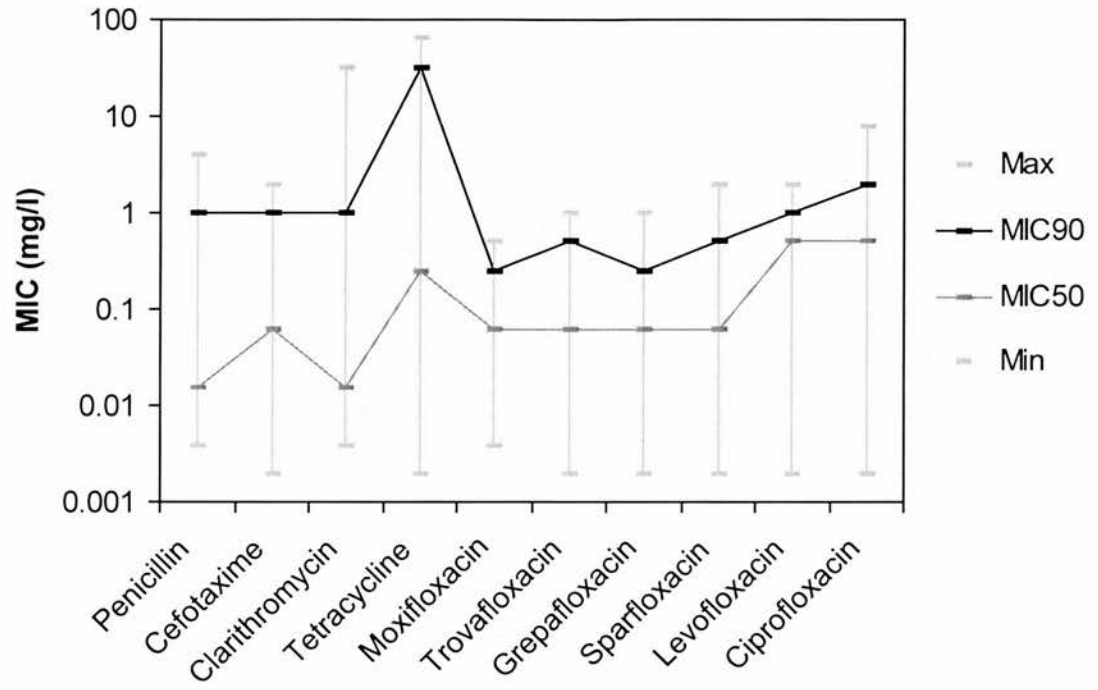


Figure 3.2: MIC_{50} and MIC_{90} of Antibiotics Tested Versus *S. pneumoniae*

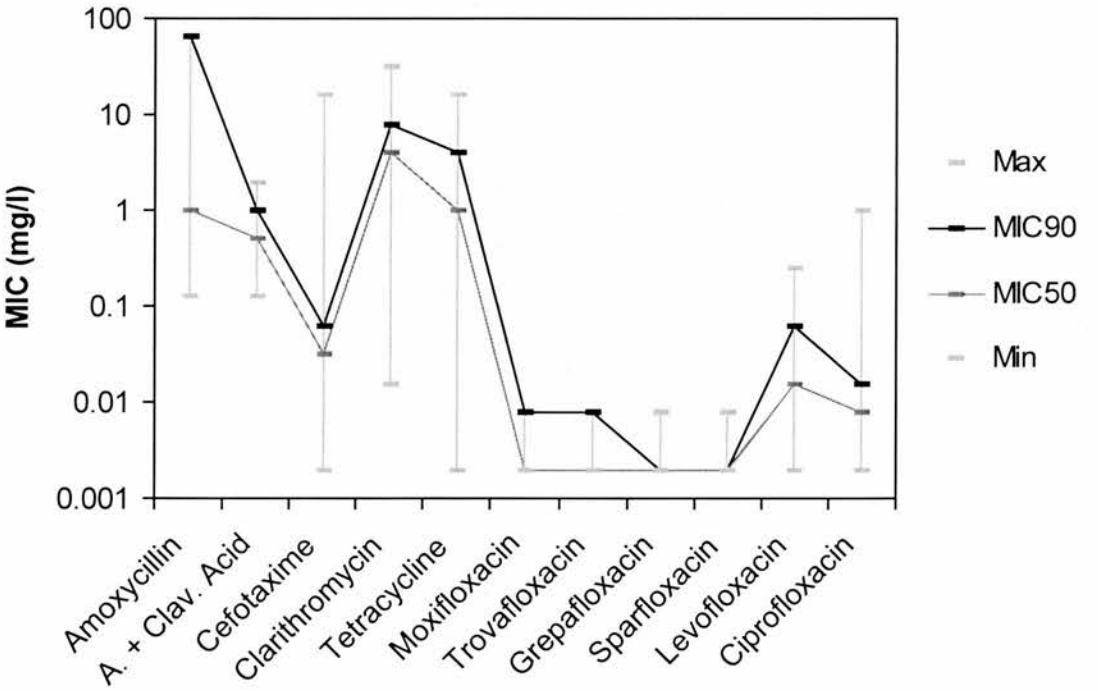


Figure 3.3: MIC_{50} and MIC_{90} of Antibiotics Tested Versus *M. catarrhalis*

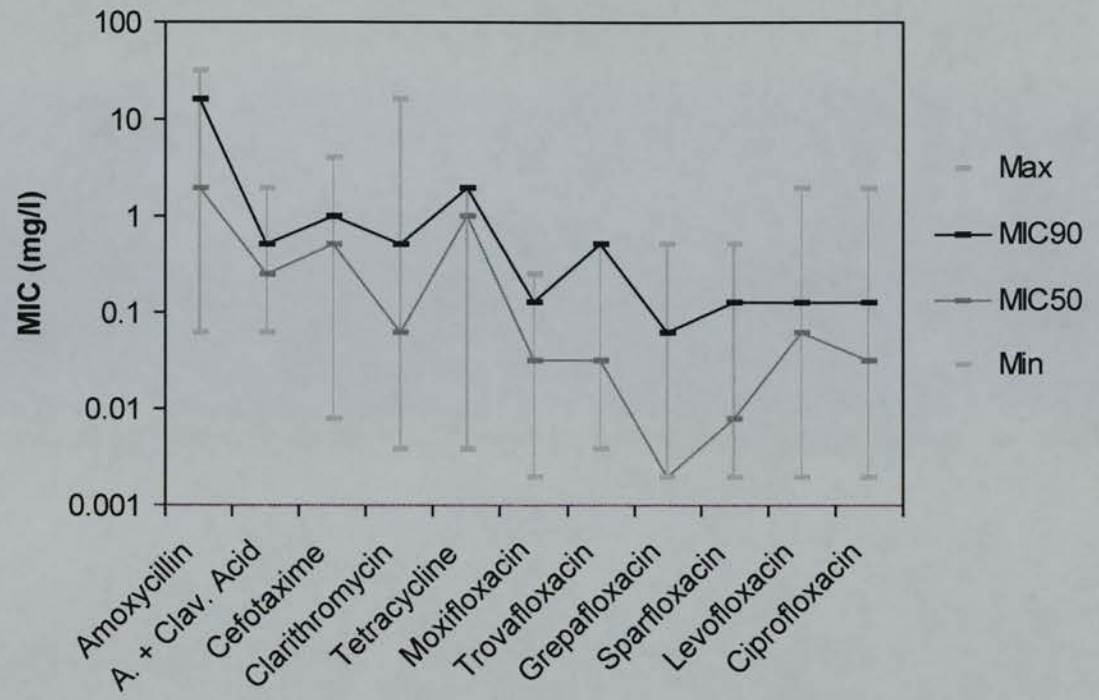


Figure 3.4: MIC_{50} and MIC_{90} of Antibiotics Tested Versus *H. influenzae*

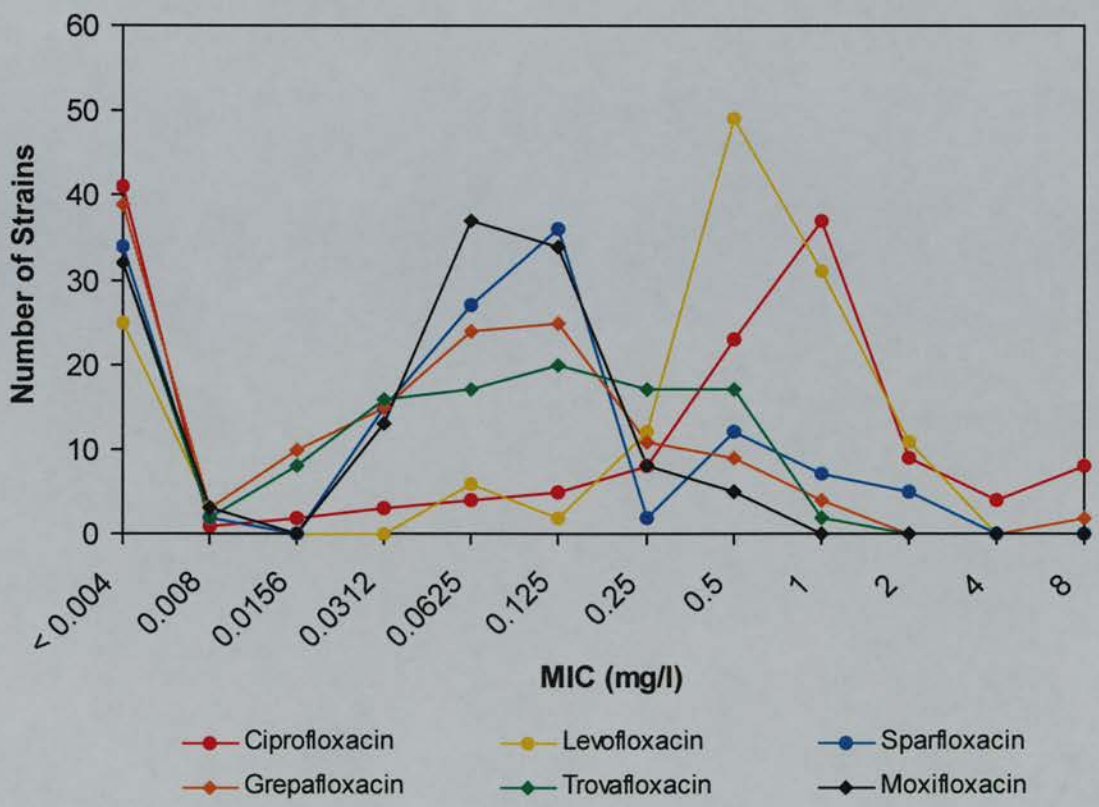


Figure 3.5: MIC Distribution of *S. pneumoniae* and Fluoroquinolones

3.3.1 Concluding Remarks

Analysis of the MIC data (Table 3.4) indicates the improved activity of the newer extended-spectrum quinolones over the older comparators against gram-positive pathogens. All three respiratory pathogens exhibit MIC_{50} and MIC_{90} below the recommended breakpoint (1mg/l) for moxifloxacin. Unlike *S. pneumoniae*, both *H. influenzae* and *M. catarrhalis* were found to be extremely sensitive to ciprofloxacin with MIC_{90} values of 0.0156mg/l and 0.125mg/l respectively. The differential activity observed between the older and the newer fluoroquinolones may be classified according to the structural modifications of the agents. This was clearly demonstrated by the increased efficacy of drugs such as moxifloxacin and trovafloxacin over ciprofloxacin and levofloxacin against *S. pneumoniae*. This distinction was not as obvious with *H. influenzae* and *M. catarrhalis* where all the fluoroquinolone agents were found to be equally effective. Generally, the activity of the quinolone class of agents surpassed that of the macrolides and the β-lactams. Tetracycline had the least activity of the antibiotics tested against both *S. pneumoniae* and *M. catarrhalis*.

3.4 Analysis of Pneumococcal Isolates with Decreased Fluoroquinolone Sensitivity

Thirty-one pneumococcal clinical isolates tested during the survey and collected during the course of this thesis that exhibited decreased susceptibility to the fluoroquinolones were further examined to elucidate the mechanism of resistance to the fluoroquinolones. Traditionally, resistance to fluoroquinolones develops by two mechanisms: alterations within target enzymes (Type II topoisomerases) or by an efflux mechanism. Initially, the sensitivity data were reconfirmed by the doubling agar dilution method to norfloxacin, ciprofloxacin, moxifloxacin and gatifloxacin. To facilitate the investigation into the efflux phenotypes of the clinical isolates, the MICs to the following established efflux pump substrates tetracycline and ethidium bromide were determined. The efflux pump inhibitor reserpine was used in efflux pump inhibition studies where the pump inhibitor was added in combination with the antibiotic. Alterations within the target genes were determined by PCR amplification of the quinolone resistance determining region of subunit A of DNA gyrase and subunits C and E of topoisomerase IV. Prior to dideoxy sequencing, the PCR products were restricted with *HinfI* and analysed by agarose gel electrophoresis. Sequenced products were subsequently compared to the quinolone susceptible strains in the GenBank database.

3.4.1 MIC Determination in the Presence of Reserpine

Of the 31 clinical isolates tested, all showed reduced susceptibility to norfloxacin (>8mg/l) and to ciprofloxacin (>1mg/l) except for one isolate. (See Figure 3.6). These strains could be divided into two groups on the basis of the effect of reserpine

on their susceptibility to norfloxacin. Group A contained four strains for which the MIC of norfloxacin was reduced only two fold or less by reserpine. Group B contained 27 strains for which the MIC of norfloxacin was reduced four-fold or greater by reserpine. The isolates in group B had susceptibility patterns similar to the norfloxacin mutants as described by Brenwald *et al* (1997; 1998).

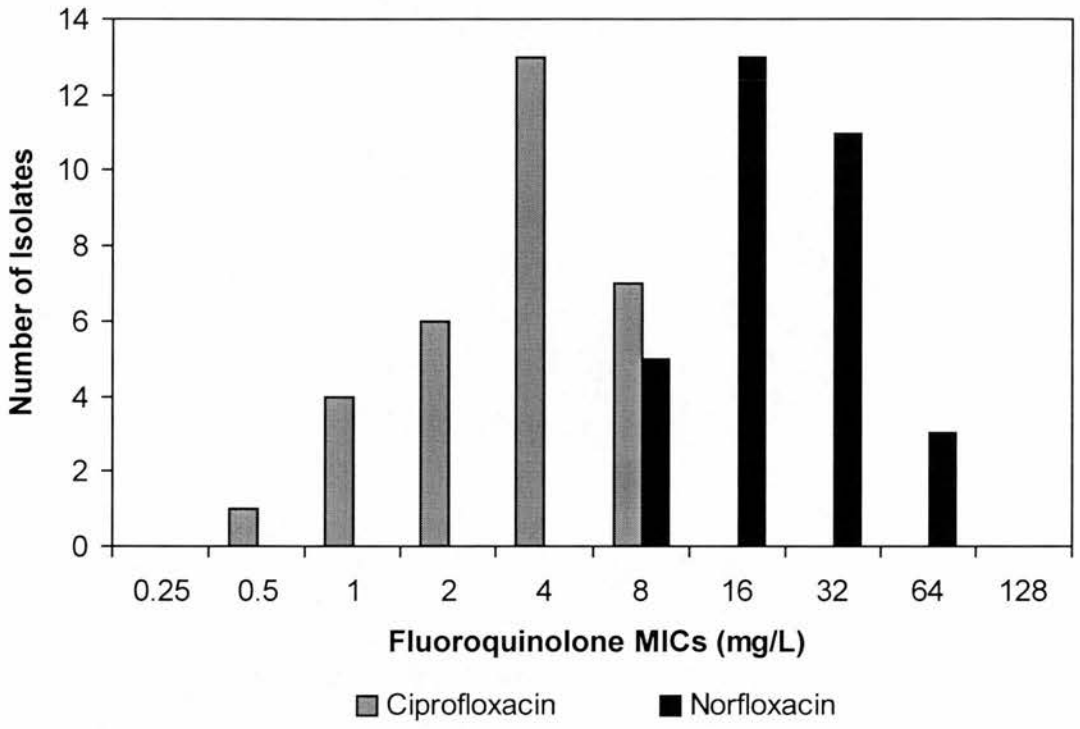


Figure 3.6: Distribution of MICs of Ciprofloxacin and Norfloxacin for 31 Clinical Isolates of *S. pneumoniae*

The ethidium bromide MICs of the clinical isolates within both groups were varied and no pattern could be established. In group A, the ethidium bromide MICs ranged between 8–32mg/l. In group B, all the 27 strains showed variations in the ethidium bromide MICs where 1 isolate had an MIC of 64mg/l. Nine strains had an MIC of 32mg/l, 12 strains at 16mg/l and 7 strains exhibited an MIC of 8mg/l. The ethidium bromide MICs of the sensitive control R6 and the efflux mutant P1Z1/IN27 were 1mg/l and 32mg/l respectively. The elevated MICs observed with the 27 strains in

group B seems to indicate that the increase could be attributed to an efflux mechanism as has been shown by Baranova *et al* (1997). Interestingly, 29 of the clinical isolates tested showed no decrease in susceptibility to tetracycline in comparison to the sensitive isolate *S. pneumoniae* R6. However, 4 strains (BPL12, A919, A12825, and A8442) exhibited extremely high MICs to tetracycline, which could possibly be attributed to a specific resistance mechanism to tetracycline.

Antibiotic	MIC Range	MIC_{50}	MIC_{90}
Norfloxacin	8 – 64	16	32
Norfloxacin + Reserpine	2 – 16	4	8
Ciprofloxacin	0.5 – 8	4	8
Ciprofloxacin + Reserpine	0.5 – 2	1	2
Moxifloxacin	0.0625 – 0.125	0.125	0.125
Moxifloxacin + Reserpine	0.0625 – 0.125	0.125	0.125
Gatifloxacin	0.25 – 0.5	0.5	0.5
Gatifloxacin + Reserpine	0.125 – 0.5	0.25	0.5
Tetracycline	0.25 – 128	0.5	64
Ethidium Bromide	8 – 64	16	32

Table 3.6: MIC_{90} of 31 Clinical Isolates with Decreased Fluoroquinolone Susceptibility

All strains were incubated overnight (18–24hr) in TH broth prior to inoculation. 18h aerobic incubation at 37°C following inoculation of columbia agar plates supplemented with 5% whole horse blood (*S. pneumoniae*) containing the appropriate concentration of antibiotic with 2µl of standardised test organism (0.5M McFarland) culture. All experiments were validated by the use of the following controls: *S. pneumoniae* P1Z1/ IN27 (See Materials &Methods), and *S. pneumoniae* R6. To ensure that reserpine had no individual inhibitory activity, agar plates containing reserpine only were inoculated to guarantee unhindered growth of the test organism. Strain P1Z1/IN27 exhibits a four-fold decrease in the norfloxacin and ciprofloxacin MIC when tested in combination with reserpine. Only 2–4 fold decreases were taken to be genuine inhibition by reserpine.

Twenty-seven strains showed a 2–8 fold decrease in ciprofloxacin MIC in the presence of reserpine. Four strains showed no reduction in the ciprofloxacin reserpine combination MICs, but demonstrated reductions when tested against the

norfloxacin reserpine combination. The MICs to both moxifloxacin and gatifloxacin against all 31 clinical isolates ranged between 0.0625mg/l – 0.125mg/l and 0.25mg/l – 0.5mg/l respectively. Both moxifloxacin and gatifloxacin MICs did not appear to be affected by the efflux pump inhibitor reserpine as no reduction was observed (See Table 3.6).

3.4.2 PCR Analysis of the Quinolone Resistance Determining Region of GyrA and ParC

Genomic DNA was isolated from overnight cultures of test organism and used as template DNA for PCR amplification of the 382bp (encoding residues 46 to 172) subunit A of DNA gyrase and the 366bp (encoding residues 36 to 157) subunit C of topoisomerase IV. Control reactions with no DNA template and with genomic DNA from *S. pneumoniae* R6 were set up to validate the experiment. All isolates were analysed through PCR and restriction digests with *Hinf*1 prior to sequencing. The absence of amplimers after the *Hinf*1 restriction digests indicates the loss of the serine active site and subsequent amino acid substitution within the QRDR. To confirm the results of the restriction digest and also to eliminate the possibility of other substitutions outwith the mutational hotspots but within the QRDR, sequence analysis of selected isolates were done. The PCR products were purified by the Qiaquick PCR purification spin columns and sequenced bi-directionally using the PCR primers. The resulting electropherograms were analysed through BLAST online search engine (http://www.ncbi.nih.gov/cgi-bin/BLAST).

3.4.2.1 Results of QRDRs of *S. pneumoniae parC* and *gyrA*

The results of the restriction digestion analysis suggested that the Serine80 site in *gyrA* and the Serine79 position in *parC* were unchanged (See Figure 3.7 & Figure 3.8). Therefore, to identify mutations outwith these sites isolates representing a range of ciprofloxacin MICs were chosen for further investigation by dideoxy sequencing. All resulting sequences were compared against the GenBank database sequences shown in Figure 3.9 and Figure 3.10.

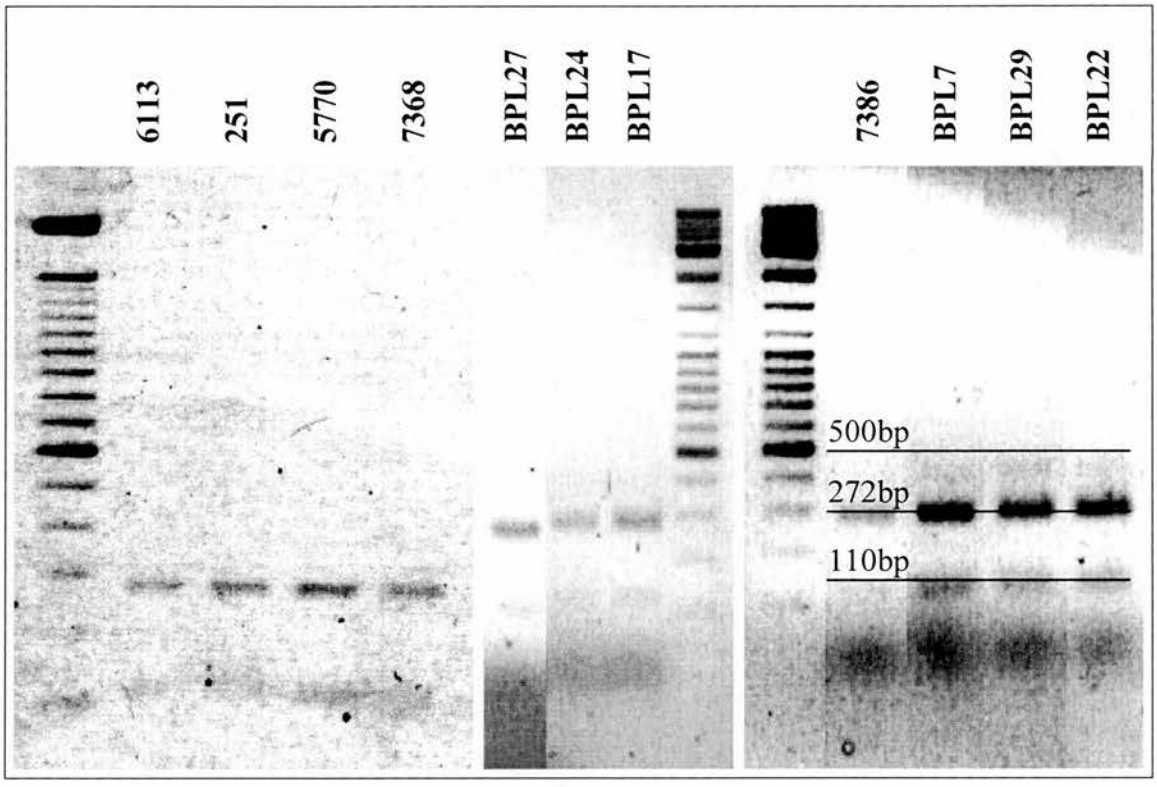


Figure 3.7: Agarose Gel Electrophoresis of Restricted Amplimers of *gyrA* PCR Products

Composite Gel photograph of Hinf1 restricted fragments of *gyrA* PCR. DNA Markers electrophoresed together with restricted PCR products are included for direct comparisons.

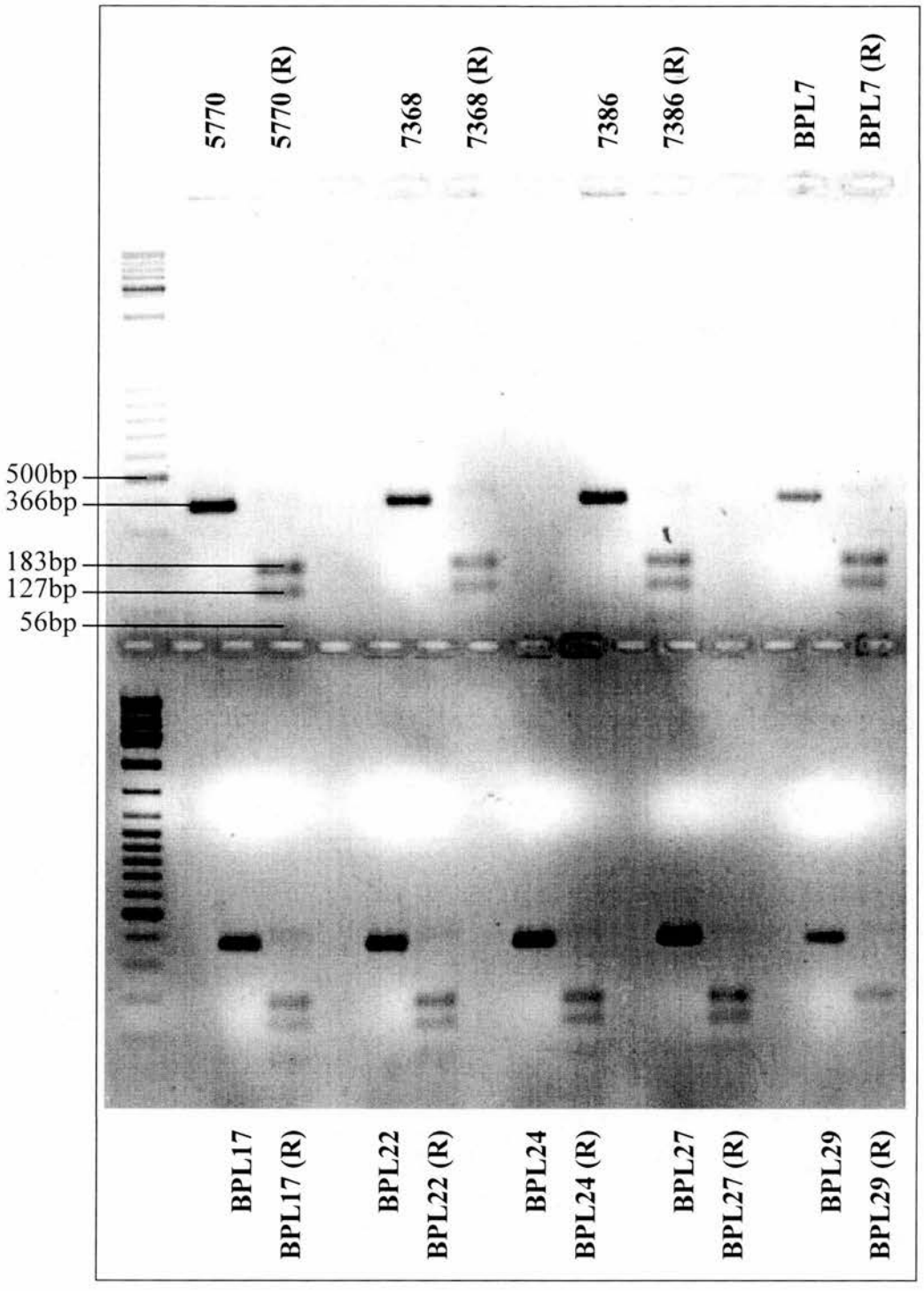


Figure 3.8: Agarose Gel Electrophoresis of *parC* PCR products and Restricted Products

Gel photograph of *parC* PCR products and restricted products from nine clinical isolates with reduced fluoroquinolone susceptibility. Isolate 6113 and 251 were directly sequenced after amplification. R indicates the *Hinf*1 restricted *parC* fragments.

```
261 c cgt cgc att ctc tat gga atg aat gaa ttg ggt gtg
      R   R   I   L   Y   G   M   N   E   L   G   V
      46
298   acc cca gac aaa ccc cat aaa aaa tct gct cgt att
      T   P   D   K   P   H   K   K   S   A   R   I

334   aca ggg gat gtc atg ggt aaa tac cac cca cac ggg
      T   G   D   V   M   G   K   Y   H   P   H   G

370   gat tcc tct att tat gaa gcc atg gtc cgt atg gct
      D   S   S   I   Y   E   A   M   V   R   M   A
          83              87
406   caa tgg tgg agc tac cgt tac atg ctt gta gat ggt
      Q   W   W   X   Y   R   Y   M   L   V   D   G

442   cat ggg aat ttt ggt tcc atg gat gga gat agt gct
      H   G   N   F   G   S   M   D   G   D   S   A

478   gcc gct caa cgt tat acc gag gca cgt atg agc aag
      A   A   Q   R   Y   T   E   A   R   M   S   K

514   att gct ctg gaa atg ctt agt gat atc aac aaa aat
      I   A   L   E   M   L   R   D   I   N   K   N

550   aca gtt gat ttc gtt gat aac tat gat gcc aat gaa
      T   V   D   F   V   D   N   Y   D   A   N   E

586   cgg gaa ccc ttg gtc ttg cca gcg cgt ttt cca aac
      R   E   P   L   V   L   P   A   R   F   P   N

622   ctt ttg gtt aat gga gca act 642
      L   L   V   N   G   A   T
                          172
```

Figure 3.9: DNA Nucleotide Sequence of a 382 bp PCR Product Encompassing the QRDR in the *gyrA* Gene from *S. pneumoniae* R6 (Accession Nu: AF053121)

Underlining highlights the *Hinf*I restriction site within the PCR fragment spanning a sequence equivalent to the Asp82 and Ser83 codons in the *E. coli gyrA*. Letters under the nucleotide sequence show the deduced protein sequence (Balas *et al*, 1998). Amino acid residues are numbered at the right by analogy with the *E. coli* GyrA protein. Nucleotide residues highlighted in bold indicate the forward and reverse primers used in the PCR.

```
105   t ggg ttg aag ccg gtt caa cgc cgt att ctt tat
        G   L   K   P   V   Q   R   R   I   L   Y
      36
139     tct atg aat aag gat agc aat act ttt gac aag
          S   M   N   K   D   S   N   T   F   D   K

172     agc tac cgt aag tcg gcc aag tca gtc ggg aac
          S   Y   R   K   S   A   K   S   V   G   N
                                                  ⇓
205     atc atg ggg aat ttc cac cca cac ggg gat tct
          I   M   G   N   F   H   P   H   G   D   S
                      ⇓                           79
238     tct atc tat gat gcc atg gtt cgt atg tca cag
          S   I   Y   D   A   M   V   R   M   S   Q
                    83
271     aac tgg aaa aat cgt gag att cta gtt gaa atg
          N   W   K   N   R   E   I   L   V   E   M

304     cac ggt aat aac ggt tct atg gac gga gat cct
          H   G   N   N   G   S   M   D   G   D   P

337     cct gcg gca atg cgt tat act gag gca cgt ttg
          P   A   A   M   R   Y   T   E   A   R   L

370     tct gaa att gca ggc tac ctt ctt cag gat atc
          S   E   I   A   G   Y   L   L   Q   D   I

403     gag aaa aag aca gtt cct ttt gca tgg aac ttt
          E   K   K   T   V   P   F   A   W   N   F

436     gac gat acg gag aaa gaa cca acg gtc ttg cca
          D   D   T   E   K   E   P   T   V   L   P

469     gca 471
          A
        157
```

Figure 3.10: DNA Nucleotide Sequence of 366 bp PCR Product Encompassing the QRDR in the *parC* Gene from *S. pneumoniae* R6 (Accession Nu: X95717)

Underlining highlights the oligonucleotides that constitute the QRDR of the *parC* gene (Pan & Fisher, 1996a). Letters under the nucleotide sequence show the deduced protein sequence. Amino acid residues are numbered at the right by analogy with the *E. coli* ParC protein. Nucleotide residues highlighted in bold indicate the forward and reverse primers used in the PCR. The amino acid residues associated with fluoroquinolone resistance have been denoted by (⇓).

Strain	MIC		Mutational Change		
	Cipro-floxacin	Moxi-floxacin	Gyr A	Par C	Par E
6113	8	0.125	—	—	IIe460 → Val
251	8	0.125	—	—	IIe460 → Val
5770	8	0.125	—	—	—
7368	4	0.125	—	Silent n/t change Gly 128 → Gly, AA change Lys 137 → Asn	—
BPL 27	4	0.125	—	Asp83 → Asn	—
BPL 24	4	0.125	—	—	—
BPL 17	4	0.125	—	—	—
7386	2	0.125	—	—	IIe460 → Val, Silent n/t change IIe476 → Ile
BPL 7	1	0.125	—	—	—
BPL 29	1	0.125	—	—	—
BPL 22	1	0.125	—	—	—

Table 3.7: Fluoroquinolone Susceptibility and Mutations in *gyrA*, *parC* and *parE* of Clinical Isolates

The strains have been sorted to show a sequential increase in ciprofloxacin MICs ranging from 1–8mg/l (See Table 3.7). From, the MIC results, it can be seen that the moxifloxacin MICs do not increase correspondingly with those of ciprofloxacin. The initial sequencing results show that none of the isolates sustain changes within the QRDR encompassing *gyrA* although the sequencing data from *parC* indicates the presence of genetic mutations within the QRDR. In isolate 7368, one silent nucleotide change, which does not result in an amino acid change and a second genuine mutation of Lysine137 → Asparagine were identified. Isolate BPL27, sustained a mutation of Aspartic acid 83 → Asparagine within the QRDR. Interestingly, isolates 6113, 251 and 5770 which exhibit MICs of 8mg/l to

ciprofloxacin did not seem to possess mutations within either *gyrA* or *parC*. All the isolates shown in Table 3.7, express an efflux phenotype to both norfloxacin and ciprofloxacin when tested in combination with the quinolone and inhibitor reserpine. Efflux hyperexpression could be attributed for the levels of ciprofloxacin MICs observed in clinical isolates 6113, 251 and 5770. Mutations within the *parE* gene have been implicated in quinolone resistance. Therefore, to further characterise the genetic mutations *parE* was amplified and sequenced. Sequence analysis of the 11 selected isolates, revealed that only 3 possessed changes within the *parE* QRDR from Isoleucine460 → Valine. One isolate 7386, also sustained a silent nucleotide substitution with no resulting amino acid change (See Table 3.7).

Most of the studies investigating the mechanisms of fluoroquinolones have attributed resistance to genetic mutations within the target genes. Recently, efflux has also been implicated to play a role in fluoroquinolone resistance development albeit contributing only towards low levels of resistance. In this study, out of the eleven isolates examined by sequencing analysis, six showed no genetic changes within the target genes. Only 1 strain (BPL 27), sustained a mutation (Asp83 → Asn) that has been demonstrated to contribute towards quinolone resistance in *S. pneumoniae* and other bacterial species (Tavio *et al*, 1999; Varon & Gutmann, 2000). The mutation of Lys137 → Asn in isolate 7368 has been reported in *S. pneumoniae* (Jones *et al*, 2000) although always in association with *gyrA* or *parC* changes and its exact contribution to fluoroquinolone resistance is yet to be established. In isolate 7368, no additional changes were found in either *gyrA* or *parC*, implying that the combination of the Lys137 → Asn mutation and efflux hyperexpression may contribute towards the elevated ciprofloxacin MIC (4mg/l). The mutation in *parE* (Ile460 → Val)

appeared in both isolates exhibiting an MIC of 8mg/l and 2mg/l thereby questioning the precise contribution of this change. In strain, 5770, no genetic mutations were identified within the QRDR despite sustaining a ciprofloxacin MIC of 8mg/l but demonstrated an efflux phenotype with ciprofloxacin and norfloxacin suggesting that efflux may contribute to higher levels of resistance than previously assumed in *S. pneumoniae*. Despite, the variable genetic changes, the common denominator amongst all the isolates investigated is the expression of the efflux phenotype with ciprofloxacin and norfloxacin.

3.5 Intracellular Targets of Moxifloxacin in *S. pneumoniae*

The effective and efficient treatment of respiratory tract infections is a crucial focus of antibiotic therapy today. *S. pneumoniae* can be identified as the single most frequent cause of respiratory tract infections in both the community and hospital (Finch, 1995). At the beginning of the antimicrobial era, *S. pneumoniae* was exquisitely sensitive to antibiotics such as penicillin and erythromycin. Since then the continued indiscriminate use of these antibiotics has led to the emergence of multi-drug resistant strains (Felmingham & Washington, 1999). New therapeutic options such as the fluoroquinolones particularly ciprofloxacin have been used leading to the subsequent emergence of fluoroquinolone resistant isolates of *S. pneumoniae*. The mechanisms by which *S. pneumoniae* develop fluoroquinolone resistance is through mutations within the target genes or by the expression of efflux mechanisms (Varon & Gutmann, 2000). The quinolone induced target mutations have only recently been molecularly characterised identifying the precise amino acid changes within both target genes i.e. *gyrA* and *parC*. These studies have shown that in *S. pneumoniae* different fluoroquinolones induce mutations within the target genes in a different order (See Table 1.2, page 60) contrary to what is observed in gram-negative bacteria, where fluoroquinolone challenge has consistently introduced mutations within DNA gyrase A prior to topoisomerase IV (Tavio *et al*, 1999).

In laboratory mutation studies involving *S. pneumoniae*, older comparators such as norfloxacin and ciprofloxacin have been shown to induce changes in *parC*, in contrast to the newer extended-spectrum quinolones, such as sparfloxacin (Pan & Fisher, 1997) and gatifloxacin (Fukuda & Hiramatsu, 1999), which introduce

mutations in *gyrA*. These results have led to the hypothesis that fundamental differences possibly exist to fluoroquinolone-induced responses between gram-positive and negative bacteria. To test this hypothesis and given the anti-pneumococcal potency of moxifloxacin against *S. pneumoniae* the primary aim of this experiment was to identify the *in vitro* target of moxifloxacin and genetically characterise the mutations that occur with moxifloxacin challenge. To ascertain the role of efflux, reserpine inhibition and accumulation studies were also done.

First-step mutants were generated by exposure of *S. pneumoniae* R6 to stepwise multiples of moxifloxacin concentrations. Subsequent mutants were selected by further challenge with the fluoroquinolone. Viable counts of mutants per plate with less than 50 cfus were determined at each concentration and also on non-selective plates so that mutation frequencies could be determined. Mutant clones were randomly selected and purified onto non-selective blood agar prior to further manipulations. The susceptibilities of the selected mutants were determined through the standard doubling agar dilution as recommended by BSAC guidelines and the genetic mutations characterised through PCR and dideoxy sequencing.

3.5.1 First Step Moxifloxacin-Selected Mutants

A total of 76 mutants spanning 5 generations were generated with serial moxifloxacin challenge. Nine first-step mutants were selected from plates containing 0.25mg/l and 0.125mg/l of moxifloxacin. The parent strain *S. pneumoniae* R6 had a moxifloxacin MIC of 0.0625mg/l and mutants were isolated from both moxifloxacin concentrations of 0.125mg/l and 0.25mg/l. Of the 9 first-step mutants selected, the increase in moxifloxacin MIC ranged between 1.3–2 times in comparison to the

parent strain. Of the 7 mutant clones selected from moxifloxacin 0.25mg/l, four possessed an MIC of 0.19mg/l, two exhibited an MIC of 0.125mg/l and one had an MIC of 0.094mg/l, which is identical to that of the parent strain. With the two clones selected from the 0.125mg/l, one retained the MIC of the parent strain R6 and other showed an increase in the moxifloxacin MIC of 1.3 times compared to R6. Two (1TS7 & 1TS9) of the four strains that exhibited the greatest increase to moxifloxacin were chosen for further study and mutations.

3.5.2 Second Generation Moxifloxacin Mutants

3.5.2.1 Second Generation Moxifloxacin Mutants Derived From 1TS7

A total of 31 strains were generated from 1TS7 with moxifloxacin challenge (See Appendix II). The mutants were generated from exposing 1TS7 to 1 (0.5mg/l), 2 (1mg/l) and 4 (2mg/l) × MIC of moxifloxacin and were isolated from plates up to 2mg/l. The increase in moxifloxacin MICs increased in the second generation mutants ranged from 0.25mg/l to 2mg/l. Mutants selected from plates containing moxifloxacin at 0.25mg/l exhibited the same moxifloxacin MICs as the parent 1TS7 and those selected from the 0.5mg/l plate sustained MICs of 0.125 – 0.25mg/l. The highest moxifloxacin MICs of 2mg/l were produced by the mutants from the plate containing the moxifloxacin concentration of 1mg/l The increase in moxifloxacin MICs in comparison to the primary parent *S. pneumoniae* R6 and parent 1TS7 are 32 times and 4 times respectively. Mutant 2TS35, which yielded the highest increase, to moxifloxacin was chosen to raise the third generation mutants.

3.5.2.2 Second Generation Moxifloxacin Mutants Derived From 1TS9

Nine mutants were isolated from moxifloxacin challenge of 1TS9 at concentrations of Mox 0.25mg/l, Mox 0.5mg/l, Mox 1mg/l and Mox 2mg/l. As seen with 1TS7, no growth was observed on the plate containing moxifloxacin 2mg/l. All the clones selected sustained MICs between 0.125mg/l–0.5mg/l. No further mutants were generated from this set.

3.5.3 Third Generation Moxifloxacin Mutants Derived From 2TS35

Mutant 2TS35 was exposed to moxifloxacin concentration ranging 4–32mg/l. No mutants were isolated from the moxifloxacin selection concentration of 32mg/l. The susceptibility profiles of the 8 selected mutants was determined by the doubling agar dilution method. The moxifloxacin MICs were found to range from 4–8mg/l. These resistant strains exhibited a 64 to 128 times increase in MIC in comparison to the primary parent *S. pneumoniae* R6 and a 2-fold increase with respect to 2TS35. Two mutants (3TS57 and 3TS58) with moxifloxacin MICs of 4 and 8mg/l respectively were selected for further mutation and analysis.

3.5.4 Fourth Generation Mutants

3.5.4.1 Fourth Generation Mutants Derived from 3TS57

Strain 3TS57 was exposed to moxifloxacin concentrations from 4–16mg/l. After the 3 day incubation period, growth was only observed on the Mox 4mg/l plate. Four clones were selected and found to exhibit an MIC of 8mg/l to moxifloxacin. A 2-fold

increase in the moxifloxacin MIC was observed for six mutant clones in comparison to the parent strain 3TS57.

3.5.5 Fifth Generation Mutants Derived from 4TS68

Strain 4TS68 was challenged with moxifloxacin concentrations ranging from 16–64mg/l. No mutant clones were isolated on moxifloxacin concentrations above that of the parent strain (8mg/l). Three of the mutant clones selected exhibited MICs above 16mg/l. No further mutants were raised from this set. All the mutant moxifloxacin MICs and the increases are presented in Appendix II.

The mutation frequencies observed with each generation do not seem to vary greatly (See Table 3.8). However, despite repeated attempts, mutant clones could not be generated on moxifloxacin concentrations above 16mg/l.

Parent Strain	Mutation Step	Mutation Frequency	Selecting Concentration (mg/l)
S. pneumoniae R6	1st	2×10^{-9}	0.5
1TS7	2nd	1.5×10^{-8}	1
1TS9	2nd	4×10^{-8}	0.5*
2TS35	3rd	2×10^{-9}	4*
3TS57	4th	6×10^{-9}	4*
4TS68	5th	3×10^{-9}	16

Table 3.8: Mutation Frequencies of All Parent Strains

Legend: * indicate that the mutant colonies were chosen from selection plates of the parent strain MICs. The viable counts and mutation frequencies of the first, second, third, fourth and fifth generation mutants were determined. The mutation frequency is determined by the following formula: MF = N / VC, where MF = Mutation Frequency, N = Number of mutants, VC = Viable count of bacteria in 100µl of overnight culture.

3.5.6 Characterisation of Moxifloxacin Induced Mutations in DNA Gyrase and Topoisomerase IV in *S. pneumoniae* R6

Moxifloxacin derived mutants were genetically characterised by both PCR and sequencing. Mutant clones were identified by means of identical MICs to the fluoroquinolones tested. Some of the mutants that were selected exhibited a decrease in the MIC to below that of the parent strain or that of the antibiotic selecting plate. The MIC ranges of the 5 mutant generations are shown in the Table 3.9 below. Mutants for further characterisation were selected from each representative groups.

Generation	MIC Range	
	Moxifloxacin	**Ciprofloxacin**
First	0.0625 – 0.5	0.5 – 1
Second	0.5 – 4	0.5 – 8
Third	4	32 – 64
Fourth	4 – 16	32 – 64
Fifth	8 – 16	64

Table 3.9: MIC Ranges of Moxifloxacin Mutant Generations

The relationship between the primary parent *S. pneumoniae* R6 and the subsequent resistant mutants derived from it with the respective increases MICs are shown in Figure 3.11.

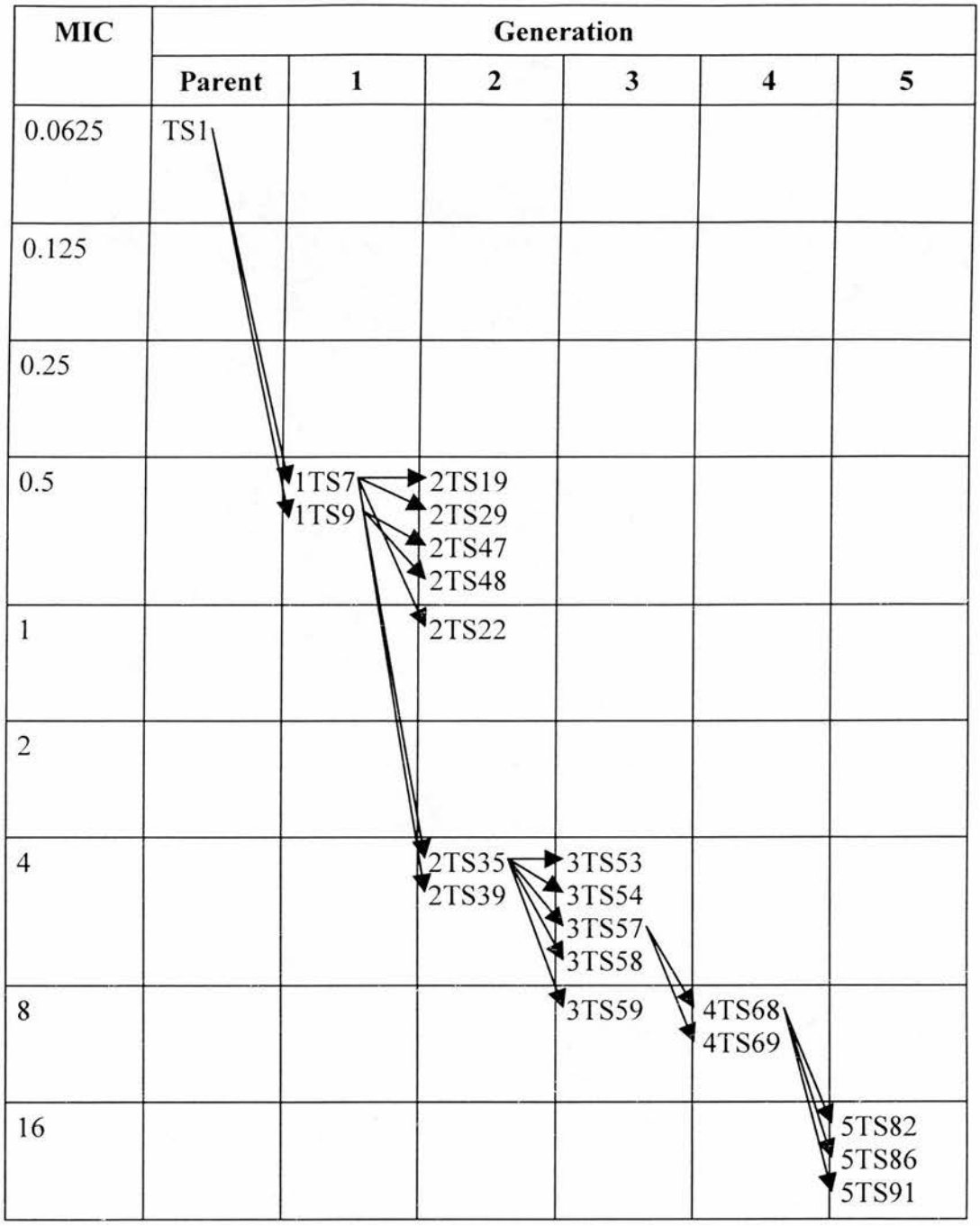


Figure 3.11: Relationship Between *S. pneumoniae* R6, Resistant Mutants and Their Respective MICs

3.5.6.1 Genetic Analysis of Quinolone Resistant Determining Region (QRDR) of Resistant Mutants

The QRDR DNA sequences of the parent strain and resistant mutants were compared to the sequence of the parent strain *S. pneumoniae* R6. Initially, the amplified

products of *gyrA* and *parC* were spliced with *Hinf*1. The 382bp *gyrA* PCR product underwent cleavage at a single *Hinf*1 (overlapping coding sequence for the conserved serine equivalent to resistance hotspot Ser83 in *E. coli*) site generating two amplimers sized at 110 and 272bp.

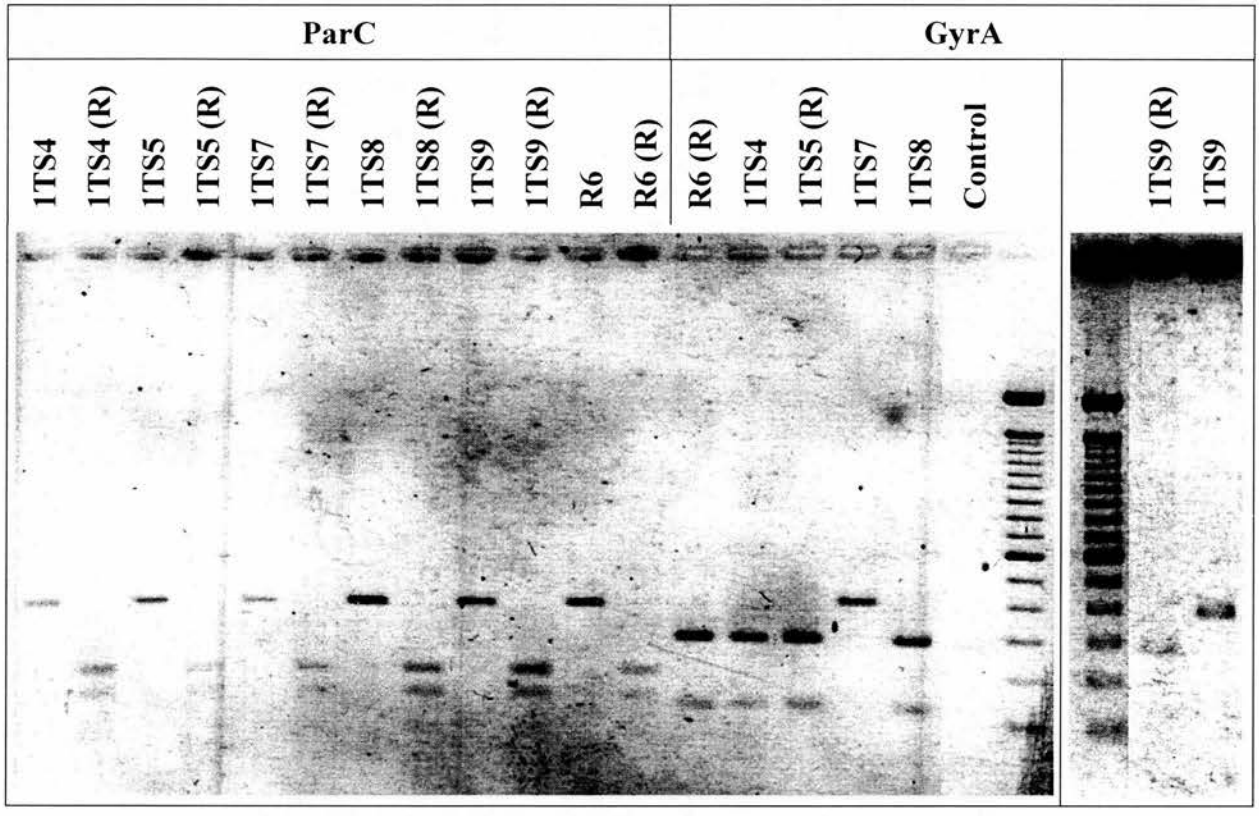


Figure 3.12: Agarose Gel Electrophoresis of ParC and GyrA PCR Products of Selected First Step Mutants Derived from *S. pneumoniae* R6

R indicates the *Hinf*1 restricted PCR products.

Interestingly, eight of the *gyrA* PCR products except 1TS7, derived from *S. pneumoniae* R6, were cleaved by *Hinf*1, indicating the absence of a mutation at codon 80 or 81. The 366bp *parC* product from the wild type gene has *Hinf*1 sites at nucleotide positions 232 and 288 and on digestion with *Hinf*1 generates fragments sized at 183, 127 and 56bp. Acquisition of a quinolone resistance mutation altering the mutational hotspot Ser-79 in *parC* leads to the loss of the 232 restriction site and

*Hinf*I cleavage resulting in the generation of two 183bp fragments. The *parC* PCR product of all first generation mutants (1TS2–1TS11) retained the restriction site, which is consistent with the absence of Ser-79 changes (See Figure 3.12). Analysis by *Hinf*I restriction fragment length polymorphism (RFLP) of the second-step mutants revealed that derivatives of 1TS7 yielded a single 183bp band suggesting an acquisition of a Ser79 change. The *parC* PCR product from the remaining second-step mutants generated from 1TS9 retained the wild type *Hinf*I digestion pattern. However, these observations do not exclude *parC* mutations outwith the enzyme recognition site. The RFLP analysis provided the evidence for a *gyrA* mutation in one first-step mutant (1TS7) and *parC* mutations in some second-step mutants derived from it. Second-step mutants generated from the other first-step mutant, 1TS9 (See Figure 3.13), sustained no *gyrA* changes.

To determine the exact amino acid change, dideoxy sequencing was done and the results are displayed in Table 3.10. One first-step mutant (1TS7) sustained a change of Serine (TCC) 80 → Tyrosine (TAC) in *gyrA*. This substitution was associated with an eight-fold decrease in moxifloxacin susceptibility. No changes were identified within the QRDR of the *parC* fragment (See Table 3.10). Subsequent mutants of 1TS7 all maintained the *gyrA* mutation and developed an additional *parC* mutation at codon 79, resulting in a change from Ser (TCT) 79 → Tyrosine (TAT). The acquisition of the *parC* mutation was associated with 16-fold increase in the ciprofloxacin MIC.

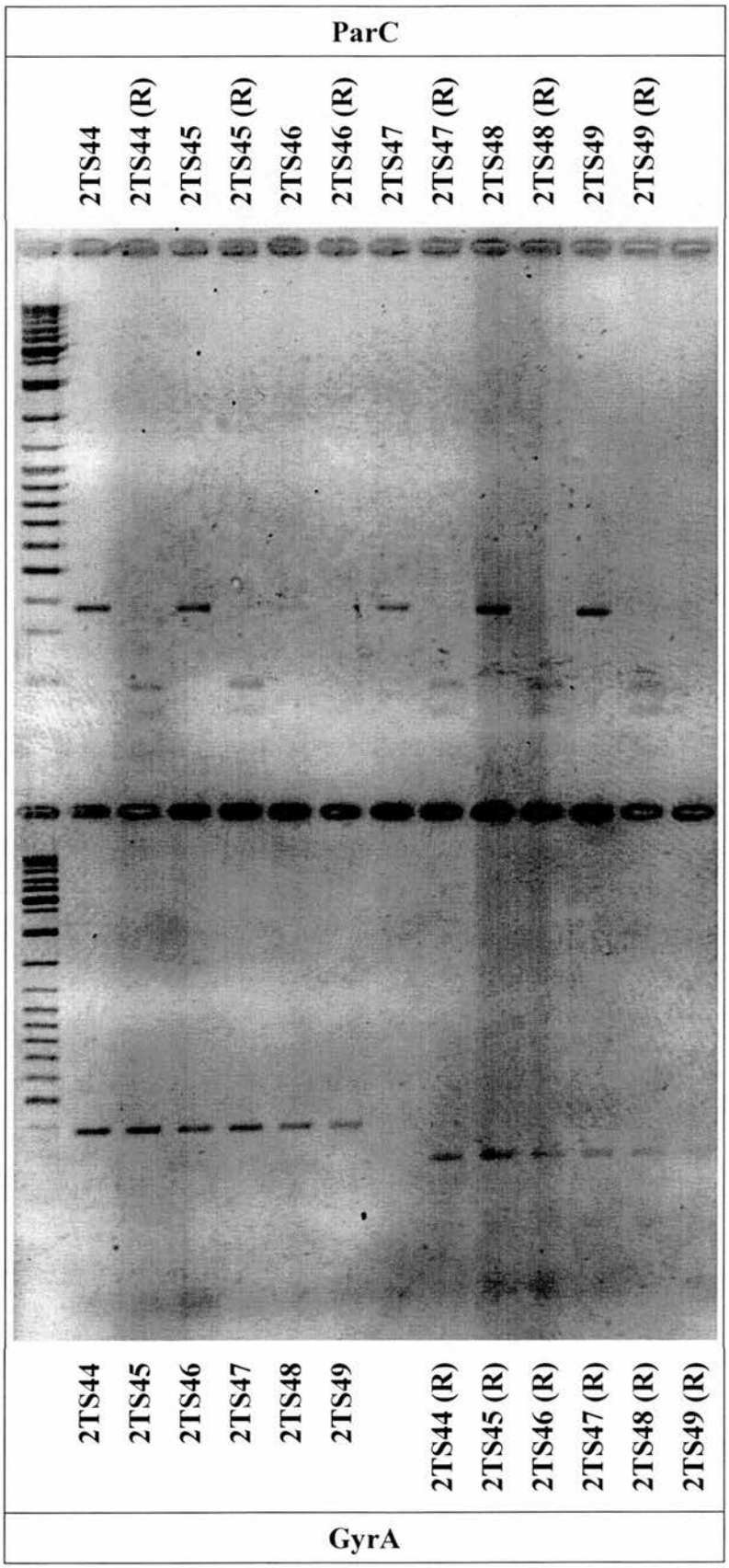


Figure 3.13: Gel Electrophoresis of Second-step Mutants Derived from 1TS9
(R) indicates the *Hinf*1 restricted PCR products.

Strain	Gyrase A	Topoisomerase IV (Subunit C)	Antibiotic MIC	
			Moxi-floxacin	Cipro-floxacin
TS1	No Change	No Change	0.0625	0.5
1TS7	Ser80 → Tyr (tcc-tac)	No Change	0.5	2
1TS9	No Change	No Change	0.5	2
2TS19	Ser80 → Tyr (tcc-tac)	No Change	0.5	1
2TS22	Ser80 → Tyr (tcc-tac)	No Change	1	1
2TS29	Ser80 → Tyr (tcc-tac)	No Change	0.5	0.5
2TS35	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	4	32
2TS39	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	4	8
2TS47	No Change	No Change	0.5	1
2TS48	No Change	No Change	0.5	2
3TS53	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	4	32
3TS54	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	4	32
3TS57	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	4	32
3TS58	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	8	32
3TS59	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	4	32
4TS68	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	8	32
4TS69	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	8	16
5TS82	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	16	64
5TS86	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tcc-tac)	16	64
5TS91	Ser80 → Tyr (tcc-tac)	Ser79 → Tyr (tct-tat)	16	32

Table 3.10: MIC Distribution and Corresponding Mutations within DNA Gyrase A and Topoisomerase IV

Interestingly, 1TS9, which exhibited the same increase in moxifloxacin MIC (0.5mg/l) as 1TS7, sustained no accompanying changes within the mutational hotspots of the either *gyrA* or *parC*. Second-step mutants derived from 1TS9 also demonstrated no substitutions within the Ser80 position as previously seen with 1TS7. Sequence analysis of the two second-step mutants 2TS47 and 2TS48

generated from 1TS9 confirmed the absence of mutations within the QRDR and correspondingly, no increases in the MIC to both ciprofloxacin and moxifloxacin were observed with either second-step mutant. No further mutants were developed from this series.

Since some of the mutants yielded no mutations within the QRDR of both *gyrA* and *parC*, quinolone mutations within the other subunits were sought. Thus, the 444bp GyrB and the 357bp ParE fragments were also analysed. Changes within *gyrB* and *parE* that are associated with quinolone resistance are Glu474 → Lys (Pan & Fisher, 1998) and Asp437 → Asn respectively (Perichon *et al*, 1997). In all the mutants generated with moxifloxacin challenge of *S. pneumoniae* R6, no changes within either *parE* or *gyrB* were identified.

3.5.7 Concluding Remarks

These results reveal two pathways of resistance development. In the first, the acquisition of a single *gyrA* mutation in mutant 1TS7 resulted in an eight-fold increase in the moxifloxacin MIC. In the second pathway, regardless of the lack of target mutations an eight-fold increase in the MIC was observed suggesting that, the induction of an efflux pump may be responsible.

3.5.8 Ciprofloxacin Selected Quinolone Resistant Mutants

The results from the previous section suggest that DNA gyrase A is the primary target of moxifloxacin in *S. pneumoniae*. This interaction could be due to the different DNA-gyrase-quinolone interactions that are formed by moxifloxacin in comparison to other quinolones e.g. ciprofloxacin. Studies have shown that variations in quinolone structures influence the differences in the *in vitro* activity against *S. pneumoniae*, which suggests that the drug-topoisomerase interactions also differ with the various agents. In order to investigate this hypothesis that different quinolones have different cellular targets, ciprofloxacin and norfloxacin were also used to generate mutants by challenging *S. pneumoniae* R6.

Ciprofloxacin selected mutants were derived from *S. pneumoniae* R6 as described before (See Chapter 2, Section 2.7.1). To analyse the order of mutations in the development of ciprofloxacin resistance, a series of ciprofloxacin resistant *S. pneumoniae* were generated. Approximately, 10^7 cfu of *S. pneumoniae* R6 (CIP MIC 0.5mg/l) were spread onto plates yielding 11 first-step mutants from the CIP 0.5mg/l selection plate and 12 mutants from the CIP 1mg/l plate (no mutants were obtained on plates containing CIP 2mg/l).

Interestingly, one first-step mutant (1C5) exhibited an MIC of 4mg/l, which was 2-fold higher than all the mutant clones selected from the CIP 1mg/l plate. Mutant 1C5 was characterised and used to select second generation mutants by further challenge with ciprofloxacin concentrations of 4, 8 and 16mg/l. Confluent growth was observed after 48 hours on both plates containing the CIP 4 and CIP 8mg/l. Five mutants were selected from both the 4 and 8mg/l plates and four mutants from the

plates containing 16mg/l. All the mutants selected from both the 4 and 8mg/l plates sustained an MIC of 2mg/l. Mutant clones selected from the plates containing 16mg/l were found to exhibit ciprofloxacin MICs of 32mg/l. Third generation mutants, were generated using the second-step mutant 2C33 (2mg/l). The third-step mutants were selected from plates containing ciprofloxacin concentrations of 8mg/l and 4mg/l. Mutant strains were characterised in terms of susceptibility to both ciprofloxacin and moxifloxacin, and the status of the QRDR of the *parC* and *gyrA* genes were ascertained by PCR and sequencing. For each of the first-step mutants, there was a minimum four-fold increase (2mg/l) and a maximum sixteen-fold increase in the ciprofloxacin MIC (8mg/l) in comparison to the parent strain. The increases in the ciprofloxacin MICs were accompanied by a two-fold increase in the moxifloxacin MIC.

3.5.8.1 Genetic Analysis of Quinolone Resistant Determining Region (QRDR) of Ciprofloxacin Resistant Mutants

First generation mutants that exhibited an MIC of 2mg/l to ciprofloxacin sustained no change within either *parC* or *gyrA*. This observation was reproducible: no *parC* alterations were detected with RFLP which was verified by the sequencing results. One first-step mutant, 1C5, which sustained an MIC of 4mg/l, underwent a mutation within the *parC* QRDR at position 79 Serine (TCT) 79 → Phenylalanine (TTT).

Analysis of other first-step mutants, with no corresponding increases in ciprofloxacin MIC, yielded no changes within the *parC* QRDR. 2C33, which was derived from mutant 1C5 retained the change within the *parC* QRDR at position 79, but with no additional changes within *gyrA*. The moxifloxacin MIC for both the selected mutants

1C5 and 2C33 was observed at 0.125mg/l and 0.25mg/l respectively (See Table 3.11). This suggests that ciprofloxacin challenge does not affect the moxifloxacin MIC. The third-step mutants sustained changes within both the *gyrA* and the *parC* QRDR. All third-step mutants and some second-step mutants that exhibited an MIC of 32mg/l for ciprofloxacin and 4mg/l to moxifloxacin were found to acquire a *gyrA* mutation of Serine80 → Tyrosine. This mutation within *gyrA* elevated the MICs to both moxifloxacin and ciprofloxacin by 16-fold and 8-fold respectively (See Table 3.11). Changes in *parE* were analysed in first-step mutants to determine its possible contribution in the development of low-level resistance to ciprofloxacin. No mutations were sustained within *parE* for all of the mutants shown in Table 3.11.

Strain	Antibiotic MIC		ParC	GyrA
	Ciprofloxacin	Moxifloxacin		
1C1	2	0.125	—	—
1C5	4	0.125	Ser79 → Phe	—
1C23	2	0.125	—	—
2C33	4	0.25	Ser79 → Phe	—
3C49	32	4	Ser79 → Phe	Ser80 → Tyr

Table 3.11: MIC Distribution and Corresponding Mutations within DNA Gyrase A and Topoisomerase IV

Number prefixes indicate mutant generation.

Interestingly, nearly all of the first-step mutants selected at CIP 2mg/l sustained no changes within the *parC* QRDR indicating that low-level resistance to ciprofloxacin may not require any mutations within the QRDR. Analysis of the QRDR status of both the *parC* and *gyrA*, it appears that the initial acquisition of *parC* mutations is the predominant route to high-level ciprofloxacin resistance.

3.5.9 Norfloxacin Selected Quinolone Resistant Mutants

The method of selection used in selecting norfloxacin resistant mutants was as described before (See Chapter 2, Section 2.7.1). The primary parent *S. pneumoniae* R6 yielded an MIC of 2mg/l to norfloxacin. Based on this, antibiotic selection plates were set up for the concentrations of 2, 4 and 8mg/l. At norfloxacin concentrations of 2 and 4mg/l, ten mutant clones were selected from the each of the plates. Fifteen colonies were selected from the 8mg/l norfloxacin selection plate. Of the thirty-five mutants selected, 8 mutants retained the MIC of the parent strain of 2mg/l; nine were found to exhibit a two-fold increase in norfloxacin at 4mg/l, 4 strains yielded an MIC of 8 and 16mg/l respectively. The mutational frequencies were calculated at 3.5×10^{-7} and 1×10^{-7} for norfloxacin concentrations of 4mg/l and 8mg/l. The decrease in the norfloxacin sensitivities was not accompanied by corresponding increases in the moxifloxacin MICs.

Strain	Antibiotic MIC			DNA Gyrase A	Topoisomerase IV
	Nor-floxacin	Cipro-floxacin	Moxi-floxacin		
R6/1	2	0.5	0.0625	—	—
R6/17	4	2	0.0625	—	Ser79 → Tyr
R6/25	16	2	0.0625	—	Ser79 → Phe

Table 3.12: MIC Distribution and Corresponding Mutations within DNA Gyrase A and Topoisomerase IV of First Generation Norfloxacin Mutants

RFLP analysis of the *gyrA* QRDR yielded no changes within the mutational hotspot (Serine 83) and was confirmed through dideoxy sequencing. Three mutants (R6/1, R6/17, and R6/25) were chosen for further analysis on the basis of their norfloxacin MICs. Genetic characterisation of these mutants verified the presence of mutations

within the *parC* subunit of topoisomerase IV (See Table 3.12). Two mutants R6/17 and R6/25 sustained mutations within *parC* at position 79. Interestingly, R6/17 sustained a mutation where Serine (TCT) 79 → Tyrosine (TAT) and R6/25 harboured a change where the Serine (TCT) 79 → Phenylalanine (TTT). This mutation from serine to both tyrosine and phenylalanine consists of a single nucleotide substitution.

Both the mutations in *parC* have contributed to an increase to both norfloxacin and ciprofloxacin MICs, although an eight-fold increase is observed with R6/25 that sustained the Serine80 → Phenylalanine change in comparison to R6/17 that only exhibited a two-fold increase with the tyrosine substitution. The change from serine a polar hydrophilic amino acid to phenylalanine a non-polar hydrophobic amino acid was followed by an eight-fold increase towards the selecting agent, norfloxacin indicating that hydrophobic amino acid substitutions may contribute towards a greater decrease in susceptibility profiles of mutants in comparison to the minimal increase observed with the substitution to tyrosine, which is also a polar hydrophilic amino acid like serine.

3.5.10 Concluding Remarks

It is clear from the results presented in the previous chapters and the published literature that quinolones that structurally differ induce mutational changes within the topoisomerases in a different order. The target preferences set out by moxifloxacin, ciprofloxacin and norfloxacin indicate the relative importance of cell-killing pathways mediated through the topoisomerases in *S. pneumoniae*. Of the three quinolones employed as selecting agents, moxifloxacin retains enhanced potency

against *S. pneumoniae* and incidentally selects for *gyrA* mutations. This is in contrast to ciprofloxacin, which exhibits variable activity, and norfloxacin, which has little or no activity against *S. pneumoniae* and select for *parC* mutations. All the mutation studies described above were performed with a laboratory strain *S. pneumoniae* R6. To correlate the mutations and susceptibility profiles with the clinical situation, mutation studies on clinically relevant isolates of *S. pneumoniae* were performed.

3.5.11 Moxifloxacin Generated Mutants from Penicillin Intermediate Strain *S. pneumoniae* 285

Worldwide initiatives involved in the evaluation of susceptibility profiles of clinical pneumococci have outlined the increased emergence of strains with reduced susceptibilities to both the β-lactams and macrolide agents (Felmingham & Washington, 1999). This has shifted the focus toward treatment options to another class of agents: the fluoroquinolones. The recent introduction of extended-spectrum quinolones such as moxifloxacin and gatifloxacin provide a therapeutic alternative. Studies have now shown that the current clinical populations of pneumococci exhibit unique characteristics. Firstly, an increasing number of pneumococcal strains demonstrate reduced susceptibility to penicillin and erythromycin and in some cases to ciprofloxacin. Secondly, analysis of clinical isolates with reduced fluoroquinolone susceptibility implicates the preferential expression of efflux pumps as a resistance mechanism in comparison to target mutations (Brenwald *et al*, 1998; Morrissey *et al*, 1999). The new extended-spectrum quinolones are directed toward these phenotypes in the clinical situation.

Therefore, the aim was firstly to elucidate the preferred targets of moxifloxacin action in clinical pneumococcal isolates and secondly to verify the relevance of the mutations observed with moxifloxacin challenged laboratory strain R6. Quinolone-resistant mutants were selected from clinical isolates of a penicillin intermediate isolate 285 (2mg/L) and penicillin resistant isolate 158 (8mg/L).

3.5.11.1 First Step Moxifloxacin Mutants Derived from Penicillin Intermediate *S. pneumoniae* 285

Penicillin intermediate *S. pneumoniae* 285 exhibited an MIC of 0.0625mg/l to moxifloxacin and 1mg/l to ciprofloxacin. Correspondingly, the isolate was challenged with moxifloxacin concentrations of 0.125mg/l and 0.25mg/l where 4 mutants were selected from the 0.125mg/l and 14 mutant clones from the 0.25mg/l plates respectively. The analysis of the sensitivities of the 18 first-generation mutants exhibited the following ranges to moxifloxacin (0.0625–0.25mg/l) and ciprofloxacin (1–8mg/l). All the first-generation mutants sustained a 2–8 fold increase in MIC to ciprofloxacin and 2–4 fold increase to moxifloxacin. Restriction fragment analysis of the first generation mutants with *Hinf*I indicated the absence of the loss of the serine at codon 83 (*E. coli* numbering). 285/3, which sustained the highest moxifloxacin MIC, was used to select the second-generation mutants.

3.5.11.2 Second Step Moxifloxacin Mutants Derived from Penicillin Intermediate *S. pneumoniae* 285

Second generation mutants were selected with moxifloxacin concentrations of 0.5mg/l and 1mg/l. Selected clones were subcultured and tested against moxifloxacin and ciprofloxacin. The majority of second-step mutants exhibited moxifloxacin MICs of 1mg/l except for two mutants (285/30 and 285/31) that demonstrated an MIC of 2mg/l to moxifloxacin. Both these mutants sustained an eight-fold increase in the moxifloxacin MIC in comparison to the parent 285/3. A corresponding increase in the ciprofloxacin MIC was also observed, with 285/31 yielding an MIC of 32mg/l and 285/30 had an MIC of 4mg/l. 285/31 was chosen for further analysis and also used to generate third generation mutants.

3.5.11.3 Third Step Moxifloxacin Mutants Derived from Penicillin Intermediate *S. pneumoniae* 285

Strain 285/31 was challenged with moxifloxacin concentrations of 4 and 8mg/l. Ten colonies were chosen from the moxifloxacin 4mg/l selection plate and 5 colonies from the 8mg/l plate. The mutants were tested and found to exhibit a moxifloxacin MIC range of 4–8mg/l. The ciprofloxacin MIC range was observed at 32–64mg/l for the third-step mutants. Moxifloxacin MICs of all 285 mutant series are presented in Appendix III.

MIC	Generation			
	Parent	1	2	3
0.0625	285			
0.125		285/7		
0.25		285/3	285/22 285/24	
0.5				
1			285/36	
2			285/31	
4				285/70
8				285/59 285/65 285/75

Figure 3.14: Relationship Between *S. pneumoniae* 285, Resistant Mutants and Their Respective MICs

Figure 3.14 details the relationship between the progeny and the parent strain *S. pneumoniae* 285. The moxifloxacin MIC increments observed with each stepwise quinolone challenge is shown.

Parent Strain	Mutation Step	Mutation Frequency
S. pneumoniae 285	1st	2×10^{-7}
285/3	2nd	3×10^{-7}
285/31	3rd	1.5×10^{-8}

Table 3.13: Mutation Frequencies of All Parent Strains

Legend: MF values were determined from overnight cultures of *S. pneumoniae* 285 using the following formula: MF= N / VC, where MF = Mutation Frequency, N = Number of mutants, VC = Viable count of bacteria in a 100µl of overnight culture.

The mutational frequency for each generation was determined and is shown in Table 3.13. The mutation frequency becomes higher as the mutants become more resistant.

3.5.12 Antimicrobial Susceptibilities of Mutants Selected from *S. pneumoniae* 285

To assess the activity of other fluoroquinolones against the moxifloxacin selected mutants, MICs were determined using the doubling agar dilution method. The results are presented in Table 3.14 below.

Antibiotic	MIC_{50}	MIC_{90}
Norfloxacin	32	128
Ciprofloxacin	4	64
Moxifloxacin	1	8
Gatifloxacin	2	8
Gemifloxacin	0.0625	1

Table 3.14: MIC_{50} and MIC_{90} Values of Fluoroquinolones Tested Against Moxifloxacin Challenged *S. pneumoniae* 285 Mutant Series

Norfloxacin was found to possess the least activity and gemifloxacin was the most active. Both moxifloxacin and gatifloxacin yielded identical MIC_{90} values although moxifloxacin was found to better gatifloxacin by a 2-fold dilution for the MIC_{50}. The

penicillin MICs were also determined for all the mutants to assess the effect of quinolone challenge on other antibiotic classes. No increases were observed with any of the mutants tested where, the penicillin MICs remained at 2mg/l identical to the parent strain.

3.5.13 Genetic Analysis of the QRDR of Moxifloxacin Resistant Mutants Derived from *S. pneumoniae* 285

PCR and dideoxy sequencing was done (See Table 3.15). Despite not sustaining any target mutations, first generation mutants 285/3 and 285/7 exhibited a 2–4 fold increase in moxifloxacin MICs.

Strain	DNA Gyrase	Topoisomerase IV		Antibiotic MIC	
	Subunit A	Subunit C	Subunit E	Moxi-floxacin	Cipro-floxacin
285	—	—	Val460 → Ile	0.0625	1
285/3	—	—	Val460 → Ile	0.25	2
285/7	—	—	Val460 → Ile	0.125	2
285/22	Ser80 → Tyr	—	Val460 → Ile	0.25	4
285/24	Ser80 → Tyr	—	Val460 → Ile	0.25	4
285/31	Ser80 → Tyr	—	Val460 → Ile	2	16
285/36	Ser80 → Tyr	—	Val460 → Ile	1	8
285/59	Ser80 → Tyr	Ser79 → Phe	Val460 → Ile	8	32
285/65	Ser80 → Tyr	Ser79 → Phe	Val460 → Ile	8	32
285/70	Ser80 → Tyr	Ser79 → Phe	Val460 → Ile	4	64
285/75	Ser80 → Tyr	Ser79 → Phe	Val460 → Ile	8	64

Table 3.15: MIC Distribution and Corresponding Mutations within DNA Gyrase A and Topoisomerase IV

The acquisition of a *gyrA* mutation (Ser80 → Tyr) in second-step clones 285/31 and 285/36 induced a 2–8 fold increase in the MIC to ciprofloxacin and a 4–8 fold increase to the moxifloxacin MIC. Interestingly, 2 second-step mutants 285/22 and 285/24 demonstrated no increases in MIC to moxifloxacin despite sustaining a mutation in *gyrA*.

The acquisition of the *parC* mutation of Ser79 → Phe in third-step mutants 285/59, 285/65, 285/70 and 285/75 elevated the ciprofloxacin MIC by 2–4 fold. The substitution Val460 → Ile within *parE* is unlikely to contribute to quinolone resistance as it is also harboured by the quinolone sensitive parent *S. pneumoniae* 285.

3.5.14 Moxifloxacin Generated Mutants from Penicillin Resistant Strain 158

To further analyse the effects of moxifloxacin challenge on penicillin resistant *S. pneumoniae*, a penicillin-resistant clinical isolate was challenged with stepwise multiples of moxifloxacin. Penicillin-resistant *S. pneumoniae* 158 was challenged with the moxifloxacin concentrations of 0.125mg/l, 0.25mg/l and 0.5mg/l. Mutants were selected and tested as described before. Ten mutants were chosen from the Mox0.25mg/l and fifteen colonies from the Mox0.5mg/l selection plates respectively. Of the 25 mutants clones analysed, 20 sustained an MIC of 0.125mg/l and five had an MIC of 0.0625mg/l to moxifloxacin. The parent strain 158 and four first-step mutants were chosen for further analysis (See Table 3.16).

RFLP analysis of the parent strain and four first-step mutants indicated no loss of the *Hinf*I recognition site in *gyrA* and *parC*. Sequencing data confirmed this observation and no changes were identified in any of the first-step mutants in both the *gyrA* and *parC*. None of the first generation mutants tested exhibited more than a 2-fold increase in the moxifloxacin MICs.

To ascertain the primary target of moxifloxacin action, second generation mutants were generated from 158/25. Five mutants were selected from the Mox 0.125mg/l plate and MICs were determined. Only one mutant 158/45 demonstrated an eight-fold increase in the moxifloxacin MIC. The remaining eleven mutants either maintained the same moxifloxacin MIC (0.0625mg/l) or exhibited a 2-fold increase (0.125mg/l) in comparison to the parent strain (158/25). With 158/45, the increase in

the moxifloxacin MIC, was also accompanied by a four-fold increase in the ciprofloxacin MIC.

Strain	DNA Gyrase	Topoisomerase IV		Antibiotic MIC	
	Gyrase A	Subunit C	Subunit E	Moxi-floxacin	Cipro-floxacin
Parent Generation:					
158	—	—	Val460 → Ile	0.0625	2
Generation 1:					
158/14	—	—	Val460 → Ile	0.0625	2
158/19	—	—	Val460 → Ile	0.0625	2
158/25	—	—	Val460 → Ile	0.125	2
158/30	—	—	Val460 → Ile	0.125	2
Generation 2:					
158/45	—	—	Val460 → Ile	0.5	8

Table 3.16: MIC Distribution and Corresponding Mutations within DNA Gyrase A and Topoisomerase IV

The difficulty in raising mutants was most pronounced with this mutation experiment where, selected clones from the different generations rarely exhibited more than 2-fold increases to moxifloxacin. Interestingly, the increments in MICs to both agents could not be attributed to mutations within any of the topoisomerase genes investigated.

3.5.15 Concluding Remarks

The mutation studies performed with all the pneumococcal isolates *S. pneumoniae* R6 and *S. pneumoniae* 285 indicate that in *in vitro* studies the primary intracellular target of moxifloxacin action is the *gyrA* subunit within DNA gyrase. From the results presented in this chapter, it is apparent that *gyrA* changes are crucial in the

evolution of resistance to moxifloxacin. With *S. pneumoniae* 158, no changes within the *gyrA* or *parC* subunit could be identified, implying that the increments to the quinolone MICs could be also due to mutations outwith the QRDR or through the induction of a moxifloxacin specific efflux pump.

3.5.16 Moxifloxacin Generated Mutants from Efflux Mutant P1Z1/IN27

The initial thrust of this project was to determine the primary *in vitro* target of moxifloxacin in *S. pneumoniae* R6. During the course of this work, it became apparent that a considerable proportion of clinical *S. pneumoniae* hyperexpressed the efflux pump *pmrA*. Therefore, to evaluate the effect of moxifloxacin on *S. pneumoniae* P1Z1/IN27 (*pmrA* hyper-expressing strain) mutation studies with stepwise antibiotic challenge were performed. Efflux mutant P1Z1/IN27 was a gift received from Dr. N.P. Brenwald, Department of Microbiology, City Hospital NHS Trust, Birmingham B18 7QH. P1Z1/IN27 is a derivative of *S. pneumoniae* ATCC 49619 after norfloxacin challenge. Sequence analysis of *gyrA*, *gyrB*, *parC* and *parE* QRDR confirmed the absence of sequence differences between the parent strain (ATCC 49619) and the efflux mutant P1Z1/IN27 (Gill *et al*, 1999). Phenotypically, P1Z1/IN27 exhibited increased sensitivity (4–8 fold) to norfloxacin in the presence of reserpine and also showed 4–8 fold decreases to ethidium bromide sensitivity than the parent strain ATCC 49619. Moxifloxacin challenge of this strain was done as described before with concentrations equivalent to 2, 4 and 8 times the MIC.

3.5.16.1 First and Second Step Moxifloxacin Mutants Derived from Efflux Mutant *S. pneumoniae* P1Z1/IN27

P1Z1/IN27 was challenged with Mox 0.125mg/l, 0.25mg/l and 0.5mg/l. Ten mutants were selected from the Mox0.125mg/l and Mox0.25mg/l plates respectively. Eleven colonies were chosen from the Mox0.5mg/l. The antibiotic susceptibilities of the

selected mutant clones were determined by the agar dilution method prior to other manipulations.

Second-step mutants were generated from first-step mutant Eff25. Generally, the older quinolones like norfloxacin and ciprofloxacin were found to be the least active against the moxifloxacin mutants. Of the newer quinolones tested, gemifloxacin was the most active with gatifloxacin being the least active. Despite, moxifloxacin being used as the selecting agent, the MIC_{90} values reported are four-fold lower than those for gatifloxacin (See Table 3.17).

Antibiotic	MIC Range	MIC_{50}	MIC_{90}
Norfloxacin	16 – >128	16	32
Ciprofloxacin	4 – 64	8	16
Moxifloxacin	0.0625 – 0.5	0.0625	0.5
Gatifloxacin	0.125 – 2	0.5	2
Gemifloxacin	0.0312 – 0.125	0.0312	0.125

Table 3.17: MIC_{50} and MIC_{90} Values of Fluoroquinolones Tested Against Moxifloxacin Challenged *S. pneumoniae* P1Z1/IN27 Mutant Series

3.5.17 Genetic Analysis of the QRDR of Moxifloxacin Resistant Mutants Derived from *S. pneumoniae* P1Z1/IN27

Restriction analysis with *Hinf*1 of the 382 bp *gyrA* PCR products produced amplimers for the parent strain P1Z1/IN27, Eff0 and Eff10 which were selected from Mox 0.125mg/l plate. Mutants Eff 22, Eff 25, Eff 26, Eff 28 and Eff 31 sustained no cleavage thereby, indicating the loss of the Ser83 site within *gyrA*. Restriction of the 366bp *parC* fragment produced identical patterns to that of the parent strain,

indicative of a sensitive *parC*. Sequencing analysis of the mutants are shown in Table 3.18.

Strain	DNA Gyrase	Topoisomerase IV		Antibiotic MIC	
	Gyrase A	Subunit C	Subunit E	Moxi-floxacin	Cipro-floxacin
P1Z1/IN27	—	—	—	0.0625	2
Eff 0	—	—	—	0.0625	2
Eff 10	—	—	—	0.0625	2
Eff 21	—	—	—	0.125	2
Eff 22	Ser80 → Phe	—	—	0.5	4
Eff 25	Ser80 → Phe	—	—	0.5	8
Eff 26	Ser80 → Phe	—	—	0.5	4
Eff 28	Ser80 → Phe	—	—	0.5	4
Eff 31	Ser80 → Phe	—	—	0.5	4

Table 3.18: MIC Distribution and Corresponding Mutations within DNA Gyrase A and Topoisomerase IV of Efflux Mutants

No changes were observed in either subunit of topoisomerase IV. Despite, the lack of *parC* mutations, the ciprofloxacin MIC increased by four-fold in Eff 25 and two-fold in Eff 22, Eff 26, Eff 28 and Eff 31. The acquisition of the *gyrA* mutation resulted in an eight-fold increase in the moxifloxacin MICs (See Table 3.18). This moxifloxacin MIC value was maintained by all the mutants that sustained the *gyrA* change. The substitution of serine, a polar hydrophilic molecule to phenylalanine a non-polar hydrophobic molecule differs from the other mutational changes observed where serine converts to tyrosine, also a polar hydrophilic amino acid. This change has been noted to occur when *S. pneumoniae* R6 was challenged with norfloxacin. The contribution of this *gyrA* mutation in the reduction of ciprofloxacin susceptibility is clearly indicated in mutant Eff 25.

3.5.18 Concluding Remarks

The results presented here clearly show that regardless of strain phenotype moxifloxacin challenged laboratory mutants always sustain primary mutations in *gyrA* followed by *parC* changes. All the mutation studies showed that mutations within *gyrA* were able to elevate the MICs of both the older agents like ciprofloxacin and norfloxacin and newer broad-spectrum agents like moxifloxacin and gatifloxacin. The *parC* mutations that were sustained had little effect on the *in vitro* potency of moxifloxacin.

3.6 Efflux Mediated Fluoroquinolone Resistance in *S. pneumoniae*

The acquisition of fluoroquinolone resistance has been attributed to mutational changes that are sustained within the primary targets of quinolone action. In the previous sections, the contributions of these target mutations have been analysed through a series of mutation studies. However, the recent identification of the pneumococcal efflux mechanism questions the exact contributions of these target mutations in fluoroquinolone resistance development. In Section 3.4.2.1 , we have shown examples of clinical pneumococcal isolates with efflux expression and no target mutations within either *parC* or *gyrA* exhibiting reduced susceptibility to the fluoroquinolones. Therefore, to ascertain the role of efflux in the defined sets of mutants that were generated, efflux inhibition experiments, antibiotic accumulation assays and analysis of efflux gene expression were conducted.

In order to examine the efflux mechanism, a phenotypic method was used. This was based on the effect of reserpine, a known efflux inhibitor, on the activity of fluoroquinolones and on the susceptibility of the strains to unrelated efflux pump substrates such as ethidium bromide and tetracycline. The effectiveness of this method for the detection of the efflux mechanism was validated with an *in vitro* mutant, P1Z1/IN27, which exhibits decreased fluoroquinolone susceptibility reversible with reserpine, and sustains no changes within the QRDR of both topoisomerase IV and DNA gyrase.

The susceptibilities of the mutants and the respective parent isolates were determined to the fluoroquinolones individually and in combination with reserpine (10mg/l) by

the standard doubling agar dilution method. Reserpine is an extremely labile agent and thus antibiotic and reserpine containing plates were made and inoculated on the same day.

3.6.1 Efflux Profile of Moxifloxacin Selected Mutants

3.6.1.1 Efflux Profile of Mutants Selected with Moxifloxacin Challenge of *S. pneumoniae* R6.

Antibiotic	MIC_{50}	MIC_{90}
Norfloxacin	2	32
Norfloxacin + Reserpine	2	32
Ciprofloxacin	1	32
Ciprofloxacin + Reserpine	1	32
Moxifloxacin	0.25	8
Moxifloxacin + Reserpine	0.25	4
Tetracycline	1	1
Ethidium Bromide	0.5	0.5

Table 3.19: MIC_{50} and MIC_{90} Values of 73 Moxifloxacin Challenged Mutants from *S. pneumoniae* R6

For the majority of the mutants tested, no reductions were observed with reserpine and fluoroquinolone combinations tested. No increments in the ethidium bromide and tetracycline MICs were exhibited by the mutants. Interestingly, four mutants (1TS7, 1TS9, 3TS53 and 4TS67) demonstrated an efflux phenotype with moxifloxacin (See Table 3.20). Only one mutant 1TS9 expressed a reduction in susceptibility when tested with reserpine to both moxifloxacin and norfloxacin. It has to be noted that 1TS9 does not sustain any mutations within the QRDR of any of the target genes. Four mutants (2TS26, 2TS27, 2TS28 and 5TS82) appeared to express a

reduction with the ciprofloxacin and reserpine combination and three mutants (1TS9, 2TS35 and 5TS86) exhibited an efflux phenotype with norfloxacin. One clone 2TS35 appeared to demonstrate a 2-fold increment in the moxifloxacin MIC and a four-fold reduction with norfloxacin when tested in combination with reserpine. Generally, no increases in the MICs of either tetracycline or ethidium bromide were recorded for any of the moxifloxacin mutants derived from *S. pneumoniae* R6.

Strain	Ciprofloxacin		Moxifloxacin		Norfloxacin		DNA Gyrase A	Topoisomerase IV Subunit C
		+ Reserpine		+ Reserpine		+ Reserpine		
1TS7	0.5	0.5	0.5	0.125	1	1	Ser80 → Tyr	—
1TS9	1	1	0.5	0.125	8	1	—	—
2TS26	4	1	0.25	0.25	4	4	Ser80 → Tyr	—
2TS27	2	0.5	0.25	0.25	4	4	Ser80 → Tyr	—
2TS28	2	0.5	0.25	0.25	4	4	Ser80 → Tyr	—
2TS35	32	32	2	4	64	4	Ser80 Tyr	Ser79 → Tyr
2TS46	8	2	0.5	0.5	64	16	—	—
3TS53	16	16	4	1	64	64	Ser80 → Tyr	Ser79 → Tyr
4TS67	16	16	4	1	64	64	Ser80 → Tyr	Ser79 → Tyr
5TS82	32	8	8	8	64	32	Ser80 → Tyr	Ser79 → Tyr
5TS86	32	32	8	8	64	16	Ser80 → Tyr	Ser79 → Tyr

Table 3.20: Minimum Inhibitory Concentration of Mutants Showing an Efflux Phenotype

The data presented here differ from the phenotypic profile of the pneumococcal efflux pump *pmrA*. Firstly, the lack of increase in both the tetracycline and ethidium bromide MICs and secondly, the reserpine potentiated reduction observed is not only confined to the hydrophilic quinolones such as ciprofloxacin and norfloxacin but is also extended to agents like moxifloxacin, which, indicates that the efflux pump

involved may be different from that of *pmrA* or that mutations within the *pmrA* gene are responsible for the altered substrate profile.

3.6.1.2 Efflux Profile of Mutants Selected with Ciprofloxacin and Norfloxacin Challenge of *S. pneumoniae* R6.

Both the R6 derived norfloxacin and ciprofloxacin mutants were tested against the reserpine fluoroquinolone combinations. None of the three ciprofloxacin challenged mutant generations tested, exhibited reductions with either ciprofloxacin and moxifloxacin. Against the norfloxacin and reserpine combination, only two of the ciprofloxacin-derived mutants (1C23 and 2C33) demonstrated a four-fold reduction (See Table 3.21).

Strain	Cipro-floxacin	Cipro-floxacin + Reserpine	Moxi-floxacin	Moxi-floxacin + Reserpine	Nor-floxacin	Nor-floxacin + Reserpine
1C23	0.125	0.5	0.0625	0.125	4	1
2C33	2	2	0.25	0.25	64	16
P1Z1/IN27	2	0.5	0.125	0.125	16	2

Table 3.21: MICs of Ciprofloxacin Generated Mutants Exhibiting an Efflux Phenotype

With the norfloxacin generated mutants, the results were similar to that obtained with the ciprofloxacin mutants. None of the norfloxacin mutants exhibited an efflux profile with any of the fluoroquinolones tested with reserpine. This result is in direct contrast to that reported by Brenwald *et al* (1997). Although, 2–4 fold increments were noted with the norfloxacin reserpine MICs of some mutants.

In all the susceptibility experiments, the control P1Z1/IN27 was used to validate the results and exhibited a consistent four-fold decrease to both the norfloxacin and

ciprofloxacin reserpine combinations in comparison to the fluoroquinolone only MIC. The susceptibility to both tetracycline and ethidium bromide were not determined for both sets of mutants.

3.6.1.3 Efflux Profile of Mutants Selected with Moxifloxacin Challenge of *S. pneumoniae* 285.

To determine the differences in the efflux profile of moxifloxacin selected mutants derived from laboratory strain *S. pneumoniae* R6 and clinical isolate *S. pneumoniae* 285, reserpine inhibition studies of the 285 mutant series were done.

The susceptibilities were determined as described before and the results are presented as MIC_{50} and MIC_{90} values to both the fluoroquinolones and other efflux pump substrates (See Table 3.22).

Antibiotic	MIC Range	MIC_{50}	MIC_{90}
Norfloxacin	16 – 128	32	128
Norfloxacin + Reserpine	0.5 – 16	8	16
Ciprofloxacin	2 – 64	4	64
Ciprofloxacin + Reserpine	2 – 16	2	16
Moxifloxacin	0.0625 – 8	1	8
Moxifloxacin + Reserpine	0.0625 – 8	1	8
Tetracycline	0.125 – 0.5	0.25	0.5
Ethidium Bromide	4 – 32	16	32

Table 3.22: MIC_{50} and MIC_{90} Values of 72 Moxifloxacin Challenged Mutants from *S. pneumoniae* 285

As observed before, no reserpine potentiated reduction was observed with moxifloxacin in contrast to the reserpine mediated reduction observed with both norfloxacin and ciprofloxacin. A four to 32-fold reduction was exhibited with the

norfloxacin and reserpine combination and the ciprofloxacin and reserpine combination conferred a more moderate 2–8 fold decrease in the ciprofloxacin MICs of all the mutants tested. Although, three mutants (285/42, 285/47, and 285/54) demonstrated no reduction in the ciprofloxacin reserpine MICs. Generally, the majority of the mutants maintained the tetracycline MICs of 0.25mg/l sustained by the primary parent isolate 285. However, 16 mutants were found to exhibit tetracycline MICs that was two-fold higher (0.5mg/l) and the remaining 16 sustained MICs two-fold lower than the parent strain (0.125mg/l).

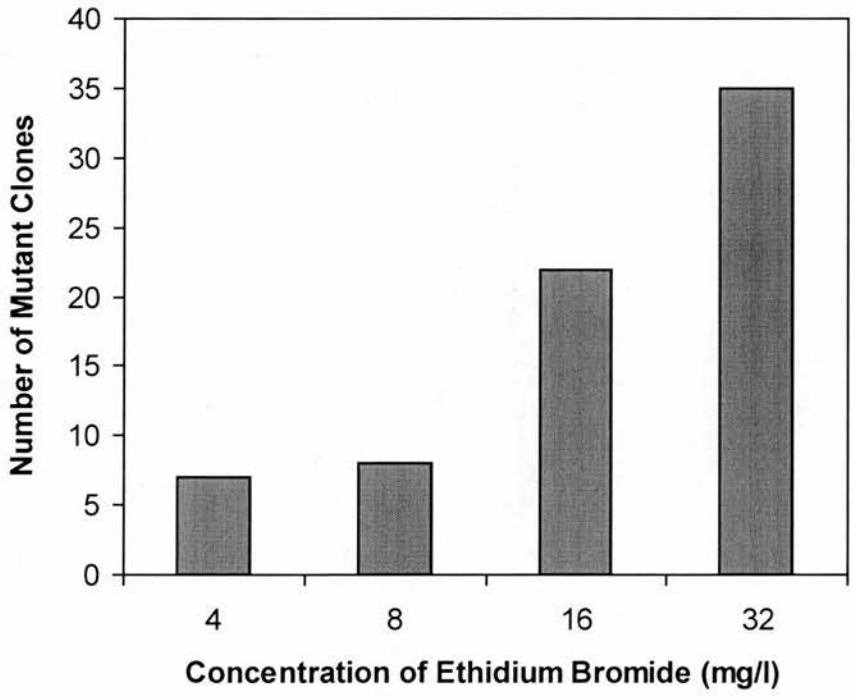


Figure 3.15: Distribution of *S. pneumoniae* 285 Derived Mutants Exhibiting Variable Ethidium Bromide MICs

Sixteen mutants retained the ethidium bromide MIC of the parent strain 285 (32mg/l). Interestingly, seven mutants were found to demonstrate an MIC of 4mg/l, and a further seven clones sustained an MIC of 8mg/l which is a 4 - 8 fold reduction in comparison to the parent strain (See Figure 3.15). All the mutants that sustained an

MIC of 4mg/l to ethidium bromide were second generation derivatives of 285/3, which had an MIC of 32mg/l. Interestingly, the mutants that exhibited a two–four fold reduction in the ethidium bromide MICs also exhibited a 2–4 fold decrease to tetracycline.

It appears that the mutants generated from the clinical isolate *S. pneumoniae* 285, exhibit a reserpine potentiated efflux phenotype with both ciprofloxacin and norfloxacin unlike that observed with the moxifloxacin resistant mutants derived from *S. pneumoniae* R6. The simultaneous MIC reduction to both tetracycline and ethidium bromide by some mutants differs from the substrate profile demonstrated by *S. pneumoniae* R6 derived mutants and the efflux pump *pmrA* (Brenwald *et al*, 1997).

3.6.1.4 Efflux Profile of Mutants Selected with Moxifloxacin Challenge of Efflux Mutant P1Z1/IN27

Constitutive expression of efflux pumps has been shown to prolong the survival of bacterial isolates by the export of antibiotics out of the cell before the intracellular targets are breached (Markham, 1999). Protection conferred by efflux pump hyperexpression has been shown to possibly precede mutations within the relevant target genes (Brenwald, *et al,* 1998; Morrissey *et al*, 1999). Therefore, to assess the contributions of the efflux pump *pmrA* in the development of moxifloxacin resistance reserpine inhibition studies were performed on the mutant series generated from *pmrA* hyperexpresser P1Z1/IN27. Efflux mutant P1Z1/IN27 was challenged with stepwise multiples of moxifloxacin, where the resulting clones sustained a mutation in *gyrA* (Ser80 → Phe) resulting in MICs of 0.5mg/l to moxifloxacin and 4mg/l to

ciprofloxacin respectively. The MIC increases can be attributed to a combination of the single *gyrA* mutation and constitutive expression of *pmrA*. To determine the exact contribution of the efflux pump in the development of fluoroquinolone resistance, MICs were done both in the absence and presence of reserpine.

Antibiotic	MIC Range	MIC_{50}	MIC_{90}
Norfloxacin	16 – >128	16	32
Norfloxacin + Reserpine	8 – 64	16	16
Ciprofloxacin	4 – 64	8	16
Ciprofloxacin + Reserpine	1 – 16	2	8
Moxifloxacin	0.0625 – 0.5	0.0625	0.5
Moxifloxacin + Reserpine	0.0625 – 0.5	0.0625	0.5
Tetracycline	0.25 – 32	0.5	1
Ethidium Bromide	16 – 32	32	32

Table 3.23: MIC_{50} and MIC_{90} Values of 49 Moxifloxacin Challenged Mutants from *S. pneumoniae* P1Z1/IN27

The parent strain P1Z1/IN27 exhibits an MIC of 0.25mg/l to tetracycline and 16mg/l to ethidium bromide. The mutants generated in this study demonstrated increases to both these efflux pump substrates. Against tetracycline, 20 mutants sustained an MIC of 1mg/l and 21 clones had an MIC of 0.5mg/l equivalent to a four-fold and two-fold increase respectively. The remaining eight mutants retained the original MIC of the parent strain. Against ethidium bromide, it was found that the MICs of some mutants decreased by two-fold to 16mg/l. Only, three mutants (Eff42, Eff43 and Eff50) were found not to sustain a reduction to the ciprofloxacin reserpine combination. Only 6 mutants (Eff 18, Eff19, Eff 24, Eff 25, Eff 49 and Eff 50) yielded a four–fold reduction with the ciprofloxacin reserpine combination. All other mutants showed a 8-fold reduction with ciprofloxacin. The efflux phenotype identified here differs

from that of the 285 mutation series where reserpine mediated reductions were observed with both ciprofloxacin and norfloxacin. Consistently, the moxifloxacin MICs for all the different mutant series showed no reduction in the presence of reserpine.

3.6.2 Concluding Remarks

The different mutation series appear to exhibit variable efflux substrate profiles. Generally, against ciprofloxacin, reductions were observed with both the 285 mutant series and the P1Z1/IN27 mutants. Norfloxacin reductions were only seen with the 285 mutant series and four of the P1Z1/IN27 series. In contrast, the *S. pneumoniae* R6 mutants derived after norfloxacin, ciprofloxacin and moxifloxacin challenge exhibited minimal reductions when tested with reserpine. The altered tetracycline and ethidium bromide profiles observed with some of mutants from both the 285 mutant series and the P1Z1 mutant series differs from that reported for *pmrA* (Brenwald *et al*, 1997) and thus, it appears that moxifloxacin challenge downregulates the effect on these agents.

3.6.3 Assay for the Accumulation of Moxifloxacin

Several studies have shown that the amount and rate of accumulation of antibiotics within the bacterial cell wall is an indication of an efflux pump hyperexpression. Inhibition studies (presented in Section 3.6.1) have shown that moxifloxacin activity is not affected by reserpine and subsequently may not be a substrate for the efflux pump *pmrA*. Given the ubiquity of efflux pumps on bacterial cells, it is possible to envisage that other efflux pumps may be involved in the uptake and export of moxifloxacin. Therefore, to ascertain the rate of moxifloxacin uptake in selected mutants, accumulation studies were done. Determination of the rate of antibiotic accumulation was performed as described by Mortimer & Piddock (1991). Briefly, centrifuged pellets of log phase bacterial cultures were resuspended in sodium phosphate buffer prior to exposure to moxifloxacin at a concentration of 10μg/ml. The decrease in fluorescence as moxifloxacin exited the cells was measured over a period of 5 minutes and recorded fluorometrically using a fluorescence spectrophotometer (excitation λ 294nm,: emission λ, 504nm) at 37°C. Selected mutants from the P1Z1/IN27 and *S. pneumoniae* R6 were analysed by the accumulation assay (See Figure 3.16 and Figure 3.17). To validate the experiment efflux mutant P1Z1/IN27 was employed.

3.6.3.1 Accumulation Assays of Mutants derived from *S. pneumoniae* P1Z1/IN27

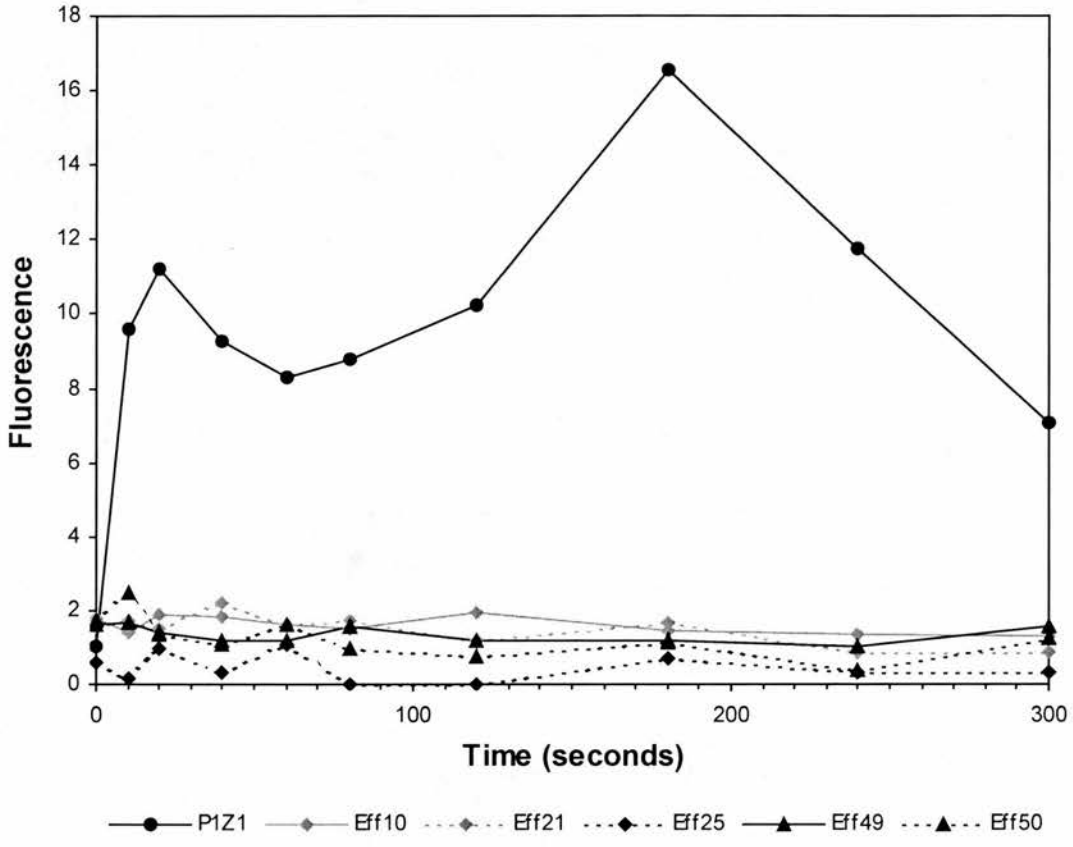


Figure 3.16: Accumulation by Parent Strain (P1Z1/IN27) and Mutant Strains (Eff10, Eff21, Eff25, Eff49 and Eff50)

Fluorescence intensity (in arbitary units) is a measure of the amount of moxifloxacin inside the cells. Graphs points were plotted as a mean of three experiments.

Figure 3.16 compares the levels of accumulation of moxifloxacin in the parent strain P1Z1/IN27 and the mutants (Eff10, Eff21, Eff25, Eff49 and Eff50) derived from it after moxifloxacin challenge. The rate of accumulation observed with the parent strain P1Z1/IN27 was found to be significantly higher than that of the mutants tested. There are no notable differences between the rate of moxifloxacin accumulation between the first generation and second generation mutants. Although, the significant decrease in the rate of accumulation that exists between P1Z1/IN27 prior to

moxifloxacin challenge and that of the mutant clones after moxifloxacin selection does indicate that an efflux pump may be a contributory factor in the decreased susceptibility of the mutants to moxifloxacin.

3.6.3.2 Accumulation Assays of Mutants derived from *S. pneumoniae* R6

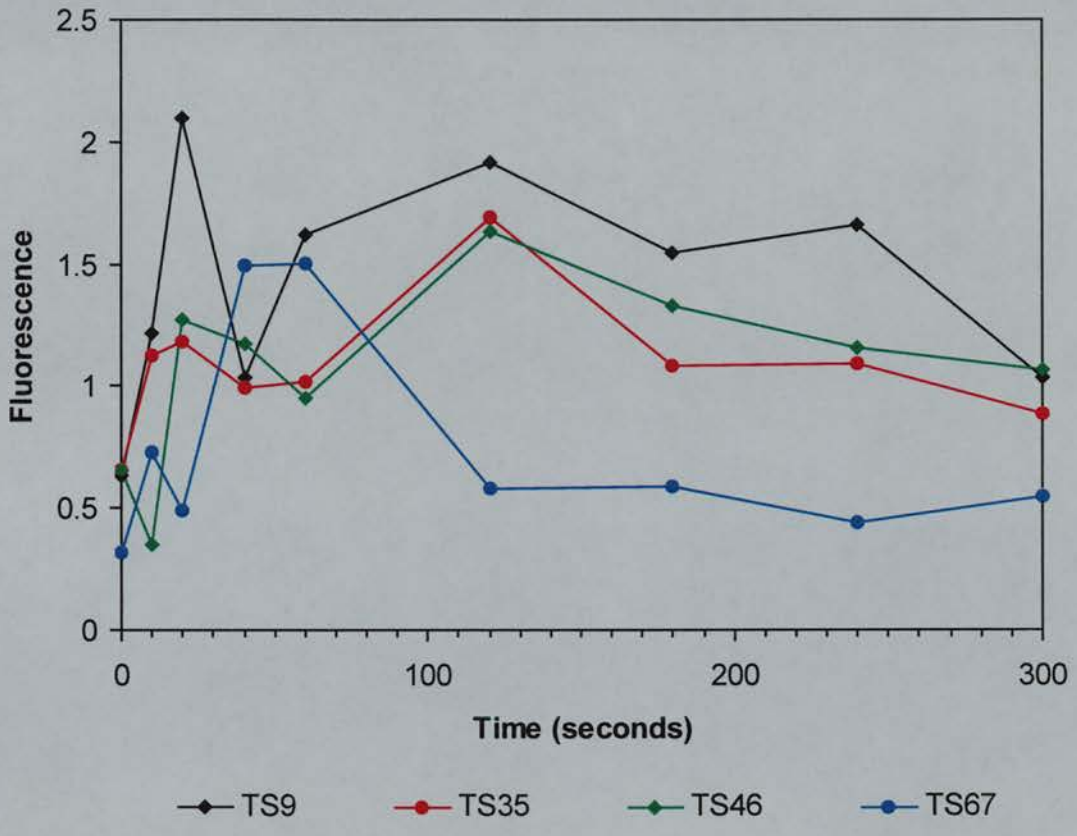


Figure 3.17: Moxifloxacin Accumulation by Selected *S. pneumoniae* R6 Mutants

Fluorescence intensity (in arbitary units) is a measure of the amount of moxifloxacin inside the cells. Graphs points were plotted as a mean of two experiments.

Mutant clones (1TS9, 2TS35, 2TS46 and 4TS67) derived from *S. pneumoniae* R6 were chosen for accumulation assay analysis (See Figure 3.17). First-step mutant 1TS9 sustained no *gyrA* or *parC* changes within the QRDR of either gene and appears to accumulate significantly higher levels of moxifloxacin than the other

mutants tested. Mutants 2TS35 and 2TS46 exhibit similar levels of moxifloxacin accumulation, despite the different target mutations sustained by both mutants (See Table 3.10). Interestingly, 4TS67 which maintains a mutation both *gyrA* and *parC* demonstrated the least levels of moxifloxacin accumulation with respect to the other mutants tested. Although 2TS35 and 4TS67 sustain the same genetic changes within both *gyrA* and *parC*, the levels of moxifloxacin accumulation of the mutants are different.

Both the accumulation assays show that moxifloxacin resistant mutants exhibit decreased levels of quinolone uptake. This effect is more pronounced in the levels observed between efflux hyper-expresser P1Z1/IN27 and the mutants derived from it. The trend observed with the *S. pneumoniae* R6 derived mutants also indicates that mutants with higher moxifloxacin MICs accumulate less quinolone. These uptake assays suggest the possibility that other efflux pump mechanisms may contribute towards the reduction in moxifloxacin uptake shown here.

3.6.4 Genetic Analysis of *pmrA* in *S. pneumoniae*

The results presented in the previous sections indicates that the phenotype of the presumptive efflux pump expressed by the mutants selected with moxifloxacin challenge is different from that of *pmrA*. In *S. aureus*, it has been shown that a single nucleotide change within the promoter region of NorA can result in either the overexpression of the efflux pump or the altered substrate profile (Ng *et al*, 1994). Therefore, to determine whether moxifloxacin exposure can induce amino acid changes within the efflux pump gene thereby altering the pump's activity and substrate specificity, sequencing analysis of the efflux pump was done. Firstly, PCR

was employed to ensure the presence of *pmrA* in all the isolates tested. Secondly, to elucidate, whether genetic changes within the efflux pump *pmrA* can alter substrate specificity and phenotypical characteristics, PCR and sequencing involving *pmrA* was performed.

Selected moxifloxacin mutants derived from *S. pneumoniae* R6 that exhibited an efflux phenotype (See Table 3.20) to moxifloxacin were analysed by PCR to establish the presence of *pmrA*. Clinical isolates of *S. pneumoniae* were also analysed to identify possible mutations within *pmrA*. The PCR was performed as described before in Chapter 2, Section 2.13 . The resulting sequences were compared against the *pmrA* sequence in the GenBank database shown in Figure 3.18.

3.6.4.1 Genetic Analysis of *pmrA* of Moxifloxacin Resistant Mutants Derived from *S. pneumoniae* R6

The *pmrA* gene was found to be present in all the mutants generated from the *S. pneumoniae* R6 (See Figure 3.19). Similarly, all the clinical isolates tested exhibited the presence of the efflux pump *pmrA*. *Cla*1 digestion of both sets of isolates indicated the resultant amplimers were consistent with that of *Cla*1 restricted *pmrA* PCR product amplified from *S. pneumoniae* P1Z1/IN27 (See Figure 3.20). Four isolates (R6, 1TS7, 1TS9, 3TS53 and 4TS67) that exhibited a reduction in the presence of reserpine was chosen for sequence analysis.

```
  30  tc aga acc agc ttc tta atc aag tgc ata tcc ttc ttg agg gag gta ggc aag

  84 atg aca gag att aac tgg aag gat aat ctg cgc att gcc tgg ttt ggt aat ttt
       M   T   E   I   N   W   K   D   N   L   R   I   A   W   F   G   N   F
 138 ctg aca gga gcc agt att tct ttg gtt gta cct ttt atg ccc atc ttc gtg gaa
       L   T   G   A   S   I   S   L   V   V   P   F   M   P   I   F   V   E
 192 aat cta ggt gta ggg agt cag caa gtc gct ttt tat gca ggc tta gca att tct
       N   L   G   V   G   S   Q   Q   V   A   F   Y   A   G   L   A   I   S
 246 gtc tct gct att tcc gcg gcg ctc ttt tct cct att tgg ggt att ctt gct gac
       V   S   A   I   S   A   A   L   F   S   P   I   W   G   I   L   A   D
 300 aaa tac ggc cga aaa ccc atg atg att cgg gca ggt ctt gct atg act atc act
       K   Y   G   R   K   P   M   M   I   R   A   G   L   A   M   T   I   T
 354 atg gga ggc ttg gcc ttt gtc cca aat atc tat tgg tta atc ttt ctt cgt tta
       M   G   G   L   A   F   V   P   N   I   Y   W   L   I   F   L   R   L
 408 cta aac ggt gta ttt gca ggt ttt gtt cct aat gca acg gca ctg ata gcc agt
       L   N   G   V   F   A   G   F   V   P   N   A   T   A   L   I   A   S
 462 cag gtt cca aag gag aaa tca ggc tct gcc tta ggt act ttg tct aca ggc gta
       Q   V   P   K   E   K   S   G   S   A   L   G   T   L   S   T   G   V
 516 gtt gca ggt act cta act ggt ccc ttt att ggt ggc ttt atc gca gaa tta ttt
       V   A   G   T   L   T   G   P   F   I   G   G   F   I   A   E   L   F
 570 ggc att cgt aca gtt ttc tta ctg gtt ggt agt ttt cta ttt tta gct gct att
       G   I   R   T   V   F   L   L   V   G   S   F   L   F   L   A   A   I
 624 ttg act att tgc ttt atc aag gaa gat ttt caa cca gta gcc aag gaa aag gct
       L   T   I   C   F   I   K   E   D   F   Q   P   V   A   K   E   K   A
 678 att cca aca aag gaa tta ttt acc tcg gtt aaa tat ccc tat ctt ttg ctc aat
       I   P   T   K   E   L   F   T   S   V   K   Y   P   Y   L   L   L   N
 732 ctc ttt tta acc agt ttt gtc atc caa ttt tca gct caa tcg att ggc cct att
       L   F   L   T   S   F   V   I   Q   F   S   A   Q   S   I   G   P   I
 786 ttg gct ctt tat gta cgc gac tta ggg cag aca gag aat ctt ctt ttt gtc tct
       L   A   L   Y   V   R   D   L   G   Q   T   E   N   L   L   F   V   S
 840 ggt ttg att gtg tcc agt atg ggc ttt tcc agc atg atg agt gca gga gtc atg
       G   L   I   V   S   S   M   G   F   S   S   M   M   S   A   G   V   M
 894 ggc aag cta ggt gac aag gtg ggc aat cat cgt ctc ttg gtt gtc gcc cag ttt
       G   K   L   G   D   K   V   G   N   H   R   L   L   V   V   A   Q   F
 948 tat tca gtc atc atc tat ctc ctc tgt gcc aat gcc tct agc ccc ctt caa cta
       Y   S   V   I   I   Y   L   L   C   A   N   A   S   S   P   L   Q   L
1002 gga ctc tat cgt ttc ctc ttt gga ttg gga acc ggt gcc ttg att ccc ggg gtt
       G   L   Y   R   F   L   F   G   L   G   T   G   A   L   I   P   G   V
1056 aat gcc cta ctc agc aaa atg act ccc aaa gcc ggc att tcg agg gtc ttt gcc
       N   A   L   L   S   K   M   T   P   K   A   G   I   S   R   V   F   A
1110 ttc aat cag gta ttc ttt tat ctg gga ggt gtt gtt ggt ccc atg gca ggt tct
       F   N   Q   V   F   F   Y   L   G   G   V   V   G   P   M   A   G   S
1164 gca gta gca ggt caa ttt ggc tac cat gct gtc ttt tat gcg aca agc ctt tgt
       A   V   A   G   Q   F   G   Y   H   A   V   F   Y   A   T   S   L   C
1218 gtt gcc ttt agt tgt ctc ttt aac ctg att caa ttt cga aca tta tta aaa gta
       V   A   F   S   C   L   F   N   L   I   Q   F   R   T   L   L   K   V
1272 aag gaa atc
       K   E   I
```

Figure 3.18: DNA Nucleotide Sequence of 1.2kb PCR Product Encompassing the *pmrA* Gene from *S. pneumoniae* P1Z1/IN27 (Accession Nu: AJ007367).

Letters under the nucleotide sequence show the deduced protein sequence. Amino acid residues are numbered at the right by analogy with the start codon ATG. Nucleotide residues highlighted in bold indicate the forward and reverse primers used in the PCR. Underlined sequence indicates the overlapping regions between the forward and reverse primer.

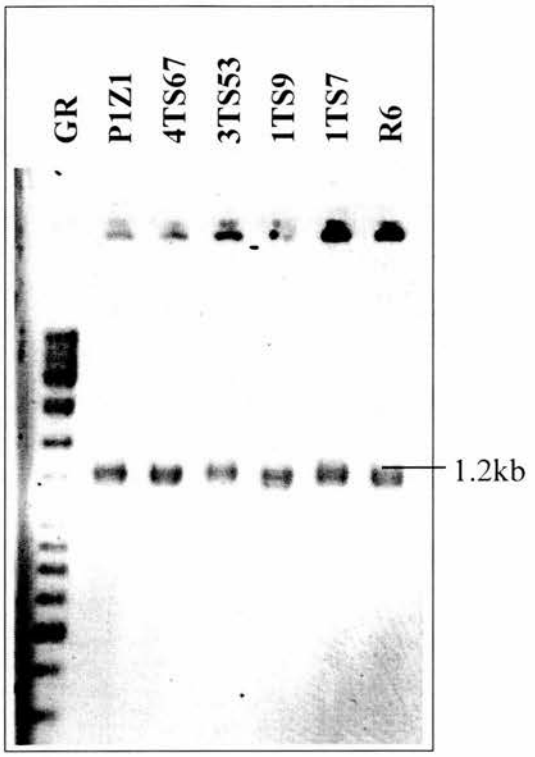


Figure 3.19: PCR Analysis Showing the Presence of *pmrA* Derived Products in *S. pneumoniae* R6 Derived Mutants

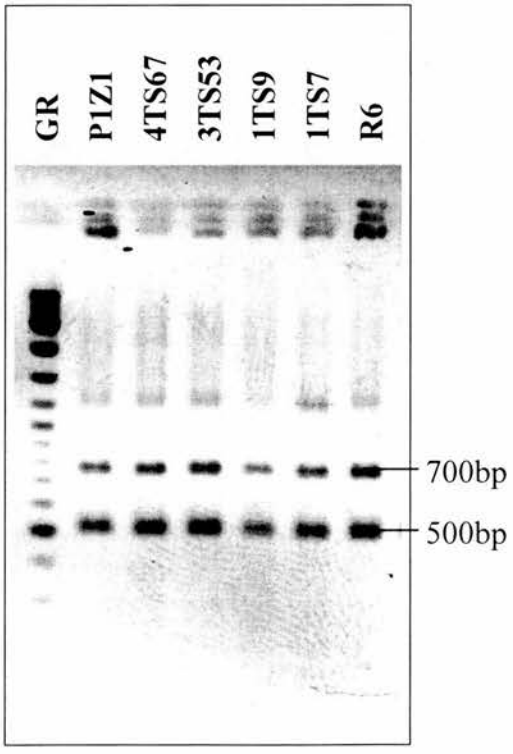


Figure 3.20: Restriction Analysis Showing the Amplimers Generated from the Cleavage of *pmrA* Derived Products

Strain	*PmrA* Change	MIC	
		Moxifloxacin	Moxifloxacin + Reserpine
R6	—	0.0625	0.0625
1TS7	—	0.5	0.125
1TS9	—	0.5	0.125
3TS53	—	4	1
4TS67	—	4	1

Table 3.24: Sequencing Results for *pmrA* Gene in Mutants Showing an Efflux Phenotype

There were no variations within the *pmrA* gene of the sensitive parent strain *S. pneumoniae* R6 and the subsequent mutants derived from it (See Table 3.24). This result indicates that no changes within the region bidirectionally sequenced can be attributed to the altered efflux phenotype observed with these mutants.

3.6.4.2 Genetic Analysis of *pmrA* in Clinical Isolates of *S. pneumoniae*

To identify any possible genetic changes within the *pmrA* gene that may contribute to decreased ciprofloxacin sensitivity in addition to or in the absence of target mutations, selected clinical isolates of *S. pneumoniae* were analysed through PCR and bi-directional dideoxy sequencing (See Figure 3.21). All relevant changes were compared to the GenBank entry of the gene *pmrA* that was cloned and sequenced from P1Z1/IN27.

No changes were observed within the 132 bp region overlap amplified by both the forward and reverse primers (See Table 3.25). Other nucleotide changes were observed with both the forward and reverse primers separately but since these were

not verified with bi-directional sequencing, the changes were not considered to be true substitutions within the efflux gene.

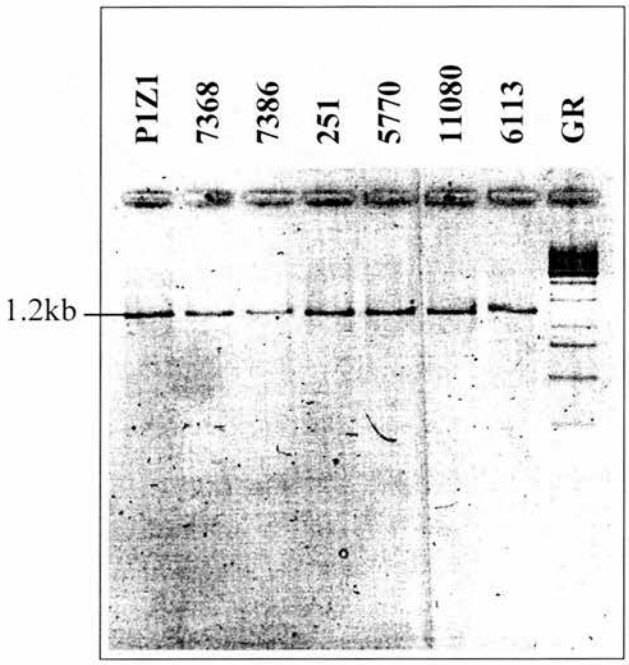


Figure 3.21: PCR Analysis Showing the Presence of *pmrA* Derived Products in Clinical Isolates of *S. pneumoniae*.

Strain	MIC		Toposiomerase Changes		
	Cipro-floxacin	Cipro-floxacin + Reserpine	GyrA	ParC	PmrA
P1Z1/IN27	2	0.5	—	—	—
7368	4	1	—	Silent n/t change Gly 128 → Gly, AA change Lys 137 → Asn	—
7386	2	0.5	—	—	—
251	8	2	—	—	—
5770	8	2	—	—	—
11080	2	0.5	—	—	—
6113	8	2	—	—	—

Table 3.25: Sequencing Results for *pmrA* Gene in Clinical Isolates with Decreased Ciprofloxacin Sensitivity

3.6.5 Northern Blot Analysis of Fluoroquinolone Resistant Mutants

The results presented in the previous sections indicate that reserpine mediated inhibition of *pmrA* increases sensitivity to the older quinolones like ciprofloxacin and norfloxacin. The data presented on *S. pneumoniae* R6 moxifloxacin mutants indicates that reserpine inhibition of the efflux pump can also result in the reduction to moxifloxacin MICs. This is in contrast to the known efflux profile of the *pmrA* pump (Brenwald *et al*, 1997; Gill *et al.* 1999). Genetic analysis of the *pmrA* pump within these moxifloxacin mutants has identified no mutations within the pumps to explain the altered substrate profile. Accumulation assays done on both the R6 and P1Z1/IN27 derived mutants have shown a decrease in uptake of the quinolone thereby indicating the involvement of an uptake barrier. Therefore, to ascertain the contribution of *pmrA* efflux expression within characterised moxifloxacin mutants, gene expression studies were done. The substrate profile of *pmrA* was further investigated by induction studies using *S. pneumoniae* R6, *S. pneumoniae* 285 and *S. pneumoniae* P1Z1/IN27. The strategy used to investigate this, was based upon the transfer of total RNA onto positively charged membrane and subsequent detection using DIG luminescent system.

3.6.5.1 Labelling of DIG Probe

PCR was employed to manufacture the probe where DIG label was incorporated into the PCR product by using a mixture of unlabelled dntps and DIG labelled dntps. The primers used to amplify the *pmrA* gene were also used in this reaction. Due to the labelled dntps, the PCR product size increased in comparison to the unlabelled probe.

The probe was digested with *Cla*1 to ensure that the correct amplimers were generated through the labelling reaction (See Figure 3.22).

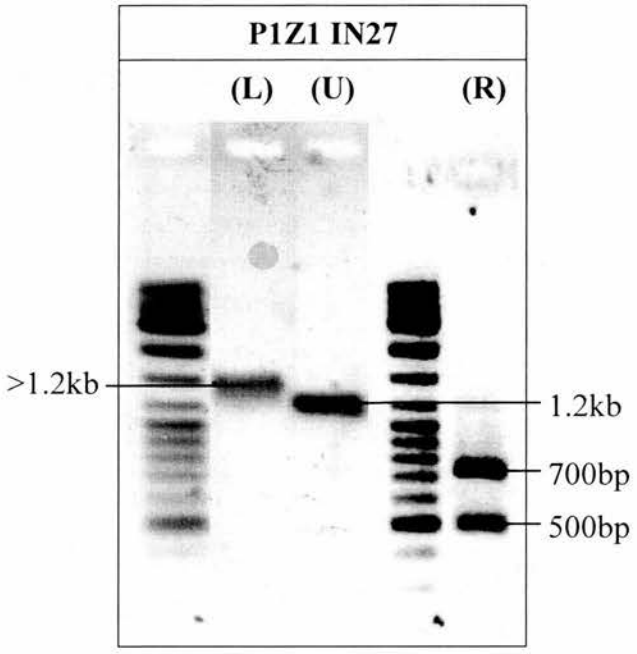


Figure 3.22: Agarose Gel Electrophoresis of P1Z1/IN27 Probe PCR Products

Agarose gel electrophoresis of labelled and unlabelled PCR products for use in the Northern blot hybridisations. Template DNA used in the PCR was derived from P1Z1/IN27. (L) denotes labelled product, (U) denotes unlabelled and (R) denotes restricted with *Cla*1.

3.6.5.2 Agarose Gel Electrophoresis of RNA samples

As described before, the RNA samples were electrophoresced on a 1% denaturing formaldehyde gel where all samples were denatured prior to loading onto the gel. Since spectophotometric standardisation of RNA samples was found to be unreliable and subsequent handling of the tubes introduced RNAses into the samples all bacterial cultures were standardised to an optical density of 0.6 at 600nm prior to RNA extraction procedures. Transfers were mounted using a downward alkaline transfer with 20 × SSC for exactly 3 hours prior to immobilisation of RNA by baking the membrane for 2 hours. Following that, all gels were stained with ethidium

bromide to ensure that the transfer of RNA from membrane to gel was complete. Hybridisation was performed overnight at 42°C, with the DNA labelled probe. Detection was done as per the manufacturer's instructions. The developed blot was visualised upon exposure to X-ray film.

3.6.5.3 Northern Blot Analysis

Three moxifloxacin mutant series were chosen for analysis (*S. pneumoniae* R6 series, *S. pneumoniae* 285 mutant series and *S. pneumoniae* P1Z1/IN27 mutant series). Total RNA extracted from both *S. pneumoniae* 285 mutant series and *S. pneumoniae* P1Z1/IN27 mutant series were separated on a denaturing formaldehyde gel (See Figure 3.23 and Figure 3.24).

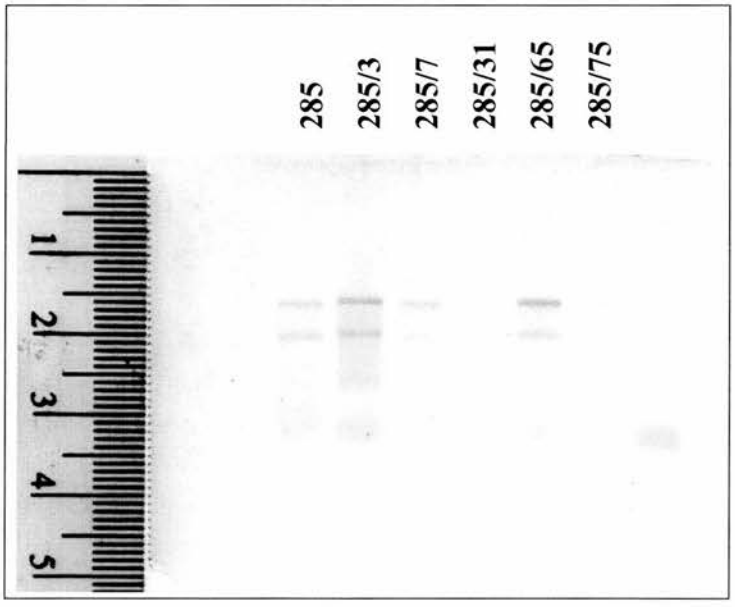


Figure 3.23: RNA Gel Electrophoresis of 285 Series Mutants

Although attempts at RNA extraction from *S. pneumoniae* R6 mutant series were successful, upon electrophoresis smears were seen instead of 2 distinct bands indicating RNA degradation. Since the methodology used in the extraction of RNA

was similar with the other mutants, other factors such as endogeneous endonucleases were considered responsible. Due to time constraints, northern blot analysis of this mutant series was not explored further.

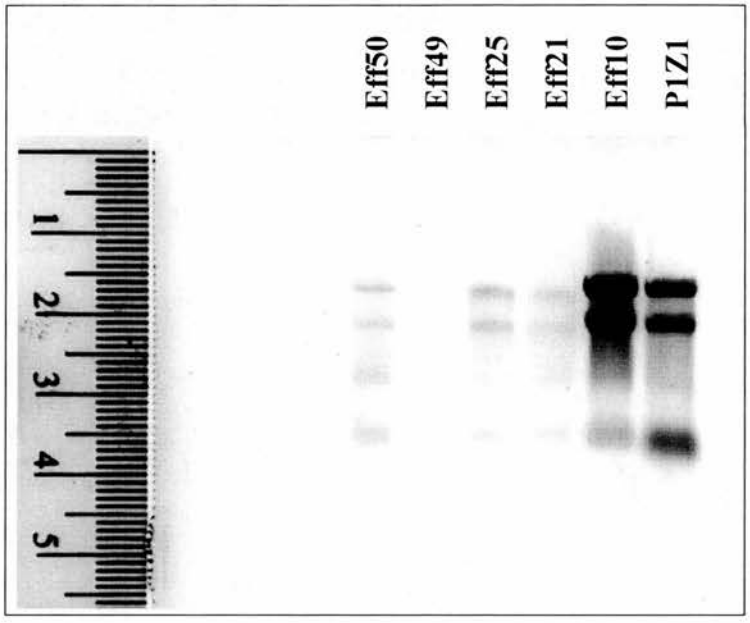


Figure 3.24: RNA Gel Electrophoresis of Efflux Mutant Series

3.6.5.4 Northern Blot Analysis of Efflux Mutant Series Derived from P1Z1/IN27

The northern blot analysis of efflux mutant series P1Z1/IN27 clearly shows that only the parent P1Z1/IN27 (*pmrA* efflux pump hyperexpresser) and first-step mutant (Eff10) have produced a positive result (See Figure 3.25). A very faint signal is observed with Eff50. All other mutants (Eff21, Eff25 and Eff49) failed to demonstrate a positive hybridisation signal with the *pmrA* probe. Thus, it is possible to suggest that moxifloxacin challenge downregulates the gene expression of *pmrA* in the more resistant mutants.

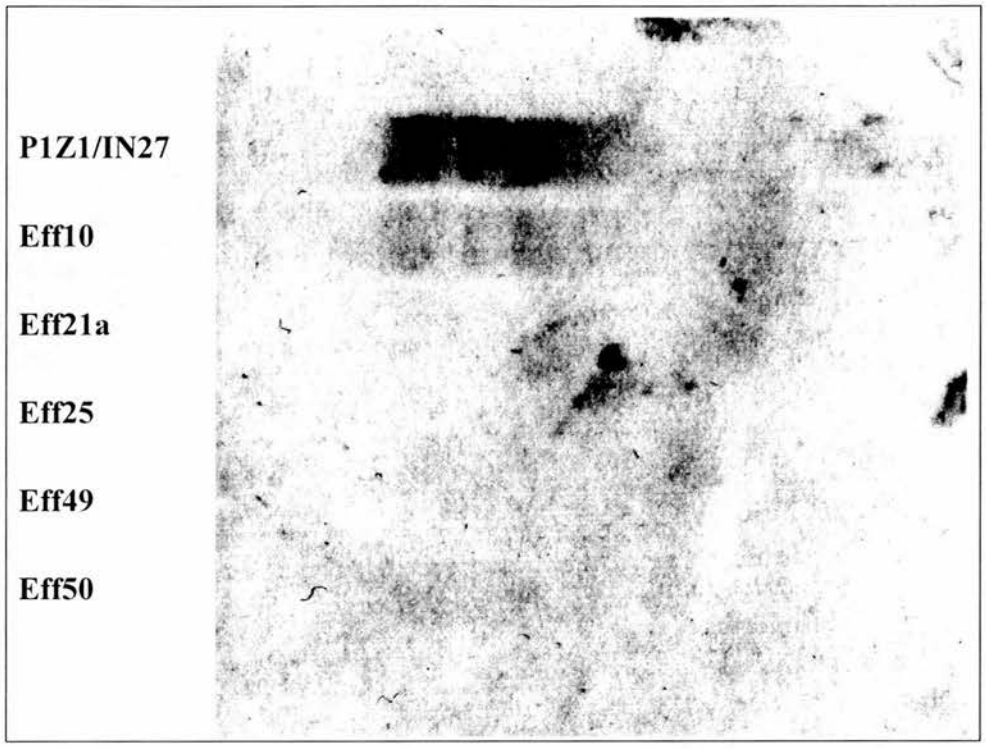


Figure 3.25: Northern Blot Analysis of Efflux Mutant Series Derived from P1Z1/IN27

3.6.5.5 Northern Blot Analysis of Mutant Series Derived from Clinical Isolate *S. pneumoniae* 285

Examination of the blot developed from *S. pneumoniae* 285 and its subsequent mutants (285, 285/3, 285/7, 285/31, 285/65 and 285/75) yielded no positive signals. The observation that no transcipted gene message was detected for the *pmrA* efflux pump gene possibly indicates either down regulation of the gene after moxifloxacin challenge or that the expression of this efflux gene is not constitutive but inducible.

However, to confirm the absence of a positive result with the 285 mutant series, a new strategy was devised. Instead of performing a gel to membrane transfer, a RNA dot blot was done. Total RNA samples were dot blotted onto positive charged membrane and probed with *pmrA* under the same conditions as described before.

3.6.5.6 Northern Dot Blot Analysis of Efflux Mutant Series Derived from P1Z1/IN27

Northern dot blot analysis showed parent strain P1Z1/IN27 and Eff10 hybridised with the probe and this observation concurred with the result obtained through northern blot analysis with one exception. Two second-step mutants (Eff49, Eff50) also produced a positive signal (See Figure 3.26). The other mutants Eff21 and Eff25 maintained the same result as in the northern blot.

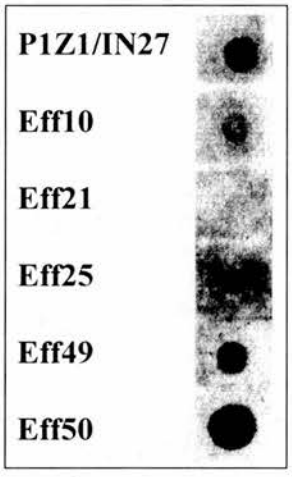


Figure 3.26: Northern Dot Blot Analysis of Efflux Mutant Series Derived from P1Z1/IN27

3.6.5.7 Northern Dot Blot Analysis of Mutant Series Derived from Clinical Isolate *S. pneumoniae* 285

The dot blot analysis confirmed the results obtained with the northern blots where no positive signals were obtained with any of the mutants (Figure not shown).

3.6.5.8 Induction Experiments with Parent Strains

To investigate whether the expression of *pmrA* was inducible or constitutive an induction study was done. The primary parent strains of each mutant series was induced at half its MIC to both ciprofloxacin and moxifloxacin prior to RNA

extraction. RNA was also obtained from the uninduced parent strains. All samples were standardised as described before. The samples were electrophoresced (See Figure 3.27) and probed. Interestingly, the electrophoresis profiles of the samples indicated that efflux mutant *S. pneumoniae* P1Z1/IN27 yielded no RNA when extracted in the presence of moxifloxacin and ciprofloxacin. Furthermore, *S. pneumoniae* R6 produced no RNA when induced with ciprofloxacin and *S. pneumoniae* 285 also yielded no RNA with moxifloxacin induction. Both RNA dot blot and northern blot analysis were done.

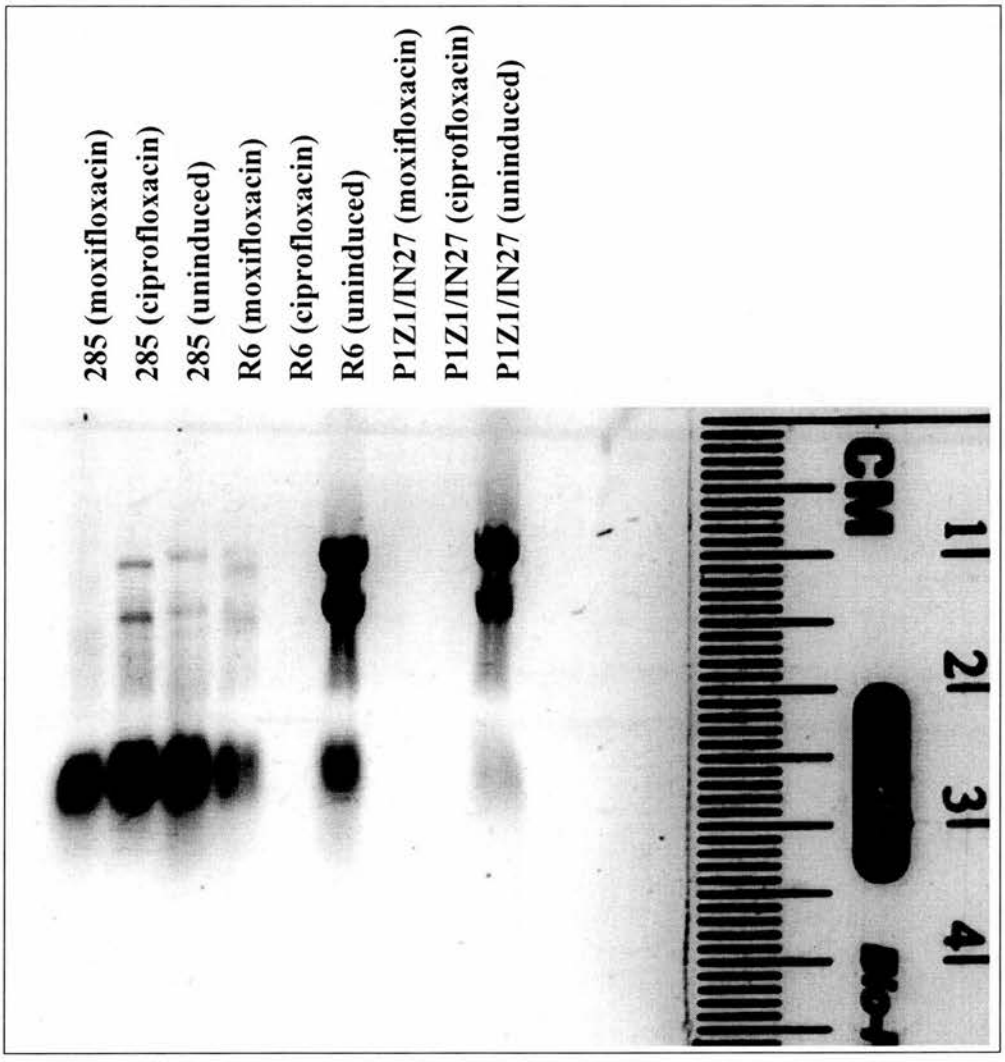


Figure 3.27: RNA Gel Electrophoresis of Induced and Uninduced Parent Strains

3.6.5.8.1 Dot Blot Analysis of Induced Parent Strains (*S. pneumoniae* R6, *S. pneumoniae* P1Z1/IN27 and *S. pneumoniae* 285)

Dot blot analysis of the three parent strains are shown in the Figure 3.28 below. All the isolates produce a positive signal with the *pmrA* probe. However, there appears to be a reduction in the intensity of the signal with the moxifloxacin induced strains. With *S. pneumoniae* R6 and *S. pneumoniae* P1Z1/IN27, the signal intensity is less pronounced in comparison to that produced by *S. pneumoniae* 285 after moxifloxacin induction. The presence of positive signals with the moxifloxacin induced strains indicates that either exposure to moxifloxacin induces the gene expression of *pmrA* or a *pmrA* like pump.

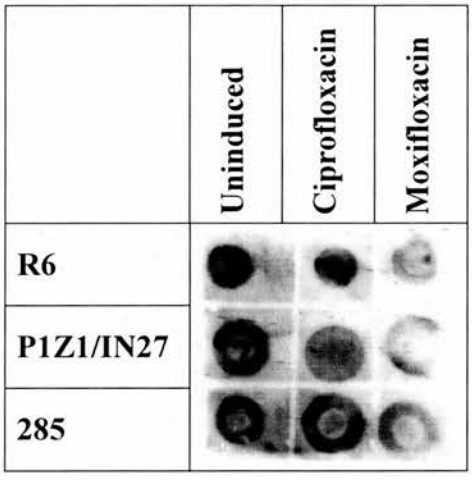


Figure 3.28: Dot Blot Analysis of Induced Parent Strains

3.6.5.8.2 Northern Blot Analysis of Induced Parent Strains (*S. pneumoniae* R6, *S. pneumoniae* P1Z1/IN27 and *S. pneumoniae* 285)

The northern blot analysis results do not concur with those of the dot blot. In this procedure, only the uninduced RNA samples from *S. pneumoniae* R6 *and S. pneumoniae* P1Z1/IN27 produced a positive hybridisation signal. *S. pneumoniae* 285 produced no hybridisation signals with both the RNA dot blot and northern blot methods (See Figure 3.29).

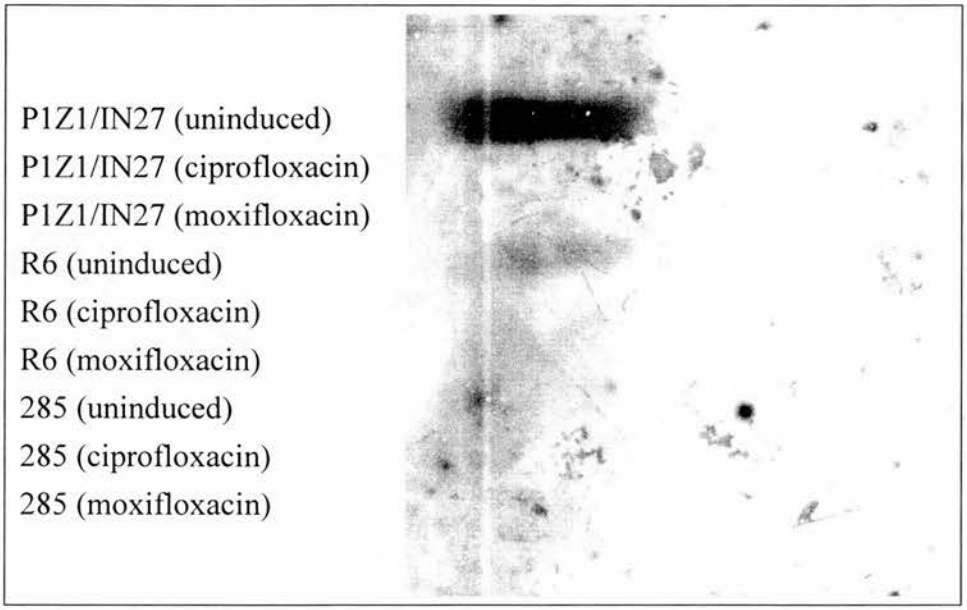


Figure 3.29: Northern Blot Analysis of Induced Parent Strains

3.6.6 Concluding Remarks

Northern blot analysis of the P1Z1/IN27 mutants series does demonstrate the absence of the relevant gene messages in first-step mutants (Eff21 and Eff25) and second-step mutant Eff49 which suggests that as the clones acquire gene mutations the efflux pump expression decreases. Due to the apparent lack of correlation between the northern blot analysis and dot blot analysis, it is difficult to draw any firm conclusions from the other experiments.

4 Discussion

4.1 Prologue

In Chapter 1, I addressed some of the questions that required answering when this project was started, together with the questions that have been raised by the progress of other researchers. The aims of this thesis were to ascertain the effects of different mutational changes as different quinolone selection pressures are applied and also the evolution of resistance mechanisms that develop with quinolone challenge in *S. pneumoniae*. The use of molecular techniques such as PCR and dideoxy sequencing clearly show that quinolone selection induces mutations within type II topoisomerases particularly within either DNA gyrase A or topoisomerase IV. Unlike gram-negative bacteria, where fluoroquinolone challenge consistently produces changes within DNA gyrase A (Tavio *et al*, 1999) gram-positive bacteria especially *S. pneumoniae* develop mutations within either *parC* or *gyrA* initially depending on

the selecting quinolone (Fukuda & Hiramatsu, 1999). The order in which these mutations arise has been an area of intense investigation, mainly to explain the differences in the chronology of mutational events that lead to quinolone resistance and also to correlate these changes to those observed within the clinical population.

The use of fluoroquinolones in the treatment of gram-positive infections particularly those caused by *S. pneumoniae* began with ciprofloxacin. However, the use of this primarily anti-gram negative quinolone against *S. pneumoniae* has in some cases led to treatment failure (Perez-Trallero *et al*, 1990) and to the subsequent emergence of ciprofloxacin resistant pneumococcal isolates (Piddock *et al*, 1998; Jones *et al*, 2000). Recent international initiatives such as the Alexander and Sentry projects aimed at monitoring the spread of antibiotic resistance, have indicated an increasing prevalence of ß-lactam or macrolide resistant pneumococci in addition to the emergence of quinolone resistant pneumococci (Felmingham & Washington, 1999). This scenario has demanded new therapeutic options and has led to the development and production of the extended-spectrum quinolones.

Moxifloxacin is a new generation quinolone that has been shown to exhibit enhanced activity against primary respiratory pathogens such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (Dalhoff *et al*, 1996; 1999). Therefore, the initial focus of this project was to examine the activity of this new quinolone against respiratory pathogens collected from centres around the United Kingdom. However, when this survey was proposed, questions regarding the testing protocols used to determine the sensitivity profiles were raised.

4.2 To Carbon Dioxide or Not To?

Generally, susceptibility testing procedures rely on the use of standardised methods that are appropriate for the species being tested. These specifications include inoculum preparation, usage of appropriate test medium and incubation conditions. The majority of the newer quinolones are targeted towards respiratory pathogens, which are traditionally isolated and tested in a carbon dioxide (CO_2) rich atmosphere. The sensitivity testing of these quinolones against respiratory pathogens has often followed the same incubation conditions despite the knowledge that the antibacterial action of some antibiotic classes may be antagonised. The BSAC guidelines (Phillips *et al*, 1991) state that carbon dioxide incubation has been shown to affect antibacterial agents such as the macrolides, quinolones and aminoglycosides. Furthermore, a survey of clinical respiratory isolates examining the activity of BAY 12-8039 (moxifloxacin) by Bruggemann *et al* (1997) demonstrated that significant increases in MIC were observed when incubations done in 5–7% CO_2 were compared with ambient air conditions. Analysis of pH values on agar surfaces indicated a significant reduction when incubated in a carbon dioxide rich atmosphere in comparison to that in air. The reduction in pH is perhaps, able to either diminish the antibacterial activity of moxifloxacin or affect the growth of the test organisms due to the low pH.

Therefore, to investigate the effect of 5% carbon dioxide incubation on the fluoroquinolones, MIC testing under normal atmospheric incubation and in a CO_2-rich atmosphere were assessed by two methods; namely the doubling agar dilution and the Epsilon test methods. The sensitivities of 18 NCTC standard strains both

traditional respiratory pathogens and hospital pathogens were measured by both sensitivity methods and under both incubation conditions. The MICs were determined, by the doubling agar dilution method and the Epsilon test, for trovafloxacin, moxifloxacin, grepafloxacin, ciprofloxacin and levofloxacin. The differences in MIC values were considered significant when 2–4 fold differences between reduced air (5% carbon dioxide) and air incubations were observed.

Increases in the quinolone MICs were observed with bacteria that are normally grown in a reduced air atmosphere as well as with those that are not. The greatest MIC increments were observed with both *S. pneumoniae* and *Ps. aeruginosa* when tested against moxifloxacin. Ciprofloxacin and moxifloxacin recorded the highest increases against *S. pneumoniae* where two to eight-fold increases in the MIC were observed when incubations were done in carbon dioxide. Against *Ps. aeruginosa*, moxifloxacin recorded a four-fold increase in the MIC when incubated in carbon dioxide in comparison to air, although similar increments were not observed with trovafloxacin and ciprofloxacin. MIC determinations recorded from the Epsilon test method concur with the results obtained with the doubling agar dilution method. If the increments observed within the 5% carbon dioxide incubations were, considered to be genuine, then NCTC strains like *S. pneumoniae* NCTC 13593 would be reclassified as being quinolone resistant.

Carbon dioxide incubation has been used in the isolation of respiratory pathogens such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* and subsequently employed in the susceptibility testing procedures involving these pathogens. Studies investigating the apparent requirement of reduced air conditions for growth have

shown that carbon dioxide incubation is not a prerequisite for growth of these organisms. A study conducted by Flanagan & Paull (1998) showed that most of the clinical pneumococci and *H. influenzae* isolates were able to grow in an aerobic environment following initial isolation in CO_2. This observation is concurred by BSAC where it was found that 40% of *S. pneumoniae* and 20% of *H. influenzae* isolates required CO_2 for initial growth (Wise, 1999). Investigators have also found that when determining the susceptibilities of these pathogens to antibiotics such as oxacillin, erythromycin, trimethoprim, tetracycline and the macrolides, accurate results were obtained in ambient air conditions rather than in 5% CO_2 (Flanagan & Paull, 1998; Weightman & Barnham, 2000).

All the studies that have evaluated the activity of antimicrobials when tested under reduced air conditions have shown that the MIC results presented after carbon dioxide incubation overestimates the number of resistant strains particularly with the macrolides in comparison to air incubation (Johnson *et al*, 1999; Flanagan & Paull, 1998; Weightman & Barnham, 2000). Furthermore, all investigators agree that reduced oxygen conditions play a role in either degrading the antibiotic or acidifying the media which compromises the action of the antibiotics tested (Jacobs, 1999; Bruggemann *et al* 1997; Phillips *et al*, 1991; Weightman & Barnham, 2000). Bruggemann *et al* (1997) were able to show a reduction in pH on the surface of the agar after carbon dioxide incubation from pH 7.6 to 6.97. Although, in this study actual pH readings were not measured, the results show substantial increases were observed in reduced air conditions in comparison to incubation in air, suggesting that the increments with 5% carbon dioxide incubations observed here may also be due to media acidification.

Some investigators argue that carbon dioxide incubation is a relevant method of testing as respiratory pathogens reside in conditions where high partial pressures of carbon dioxide are known to exist (Johnson *et al*, 1999). Therefore, evaluating activity within these conditions portrays the true activity of the antibiotic. Although this may be a valid suggestion, it has to be conceded that the dynamic *in vivo* conditions are impossible to reproduce within the *in vitro* testing conditions. Perhaps, one possibility is to modify the test media to eliminate the pH reduction factor and resolve the subsequent discrepancies observed with the carbon dioxide and air incubation methods. Studies investigating macrolide sensitivities have shown that the MIC values vary only by a maximum 2-fold difference between the air and reduced air conditions when testing was done on pH adjusted media (Johnson *et al*, 1999; Ednie *et al*, 1998).

Controlled standardised methods are essential in evaluating and producing surveillance data. Here, we have shown that carbon dioxide incubations can undermine the potency of antimicrobial compounds leading to false interpretations of susceptibility data. Although MIC determinations are not solely used in the evaluation of antibacterial activity, it is a standard procedure used in most clinical and research laboratories, therefore clear recommendations are needed especially with the recent introduction of anti-gram-positive quinolones. Carbon dioxide incubation may be a crucial component in the isolation of respiratory strains but is clearly unsuitable for susceptibility testing.

4.3 Moxifloxacin Sensitivity Study

Currently, acute respiratory tract infections (RTI) are the third-highest cause of morbidity and mortality worldwide (WHO report, 1997). Antimicrobial resistance in the causative pathogens of respiratory tract infections are increasingly common (Grüneberg *et al*, 1996). International initiatives such as the Alexander Project and the Sentry Project have identified a need for continuing surveillance data to monitor the rise in antibiotic resistance in respiratory pathogens. Both studies have clearly outlined a rise in antimicrobial resistance within respiratory isolates and regional variations in resistance patterns have been shown to exist (Baquero *et al*, 1991).

Although the surveillance data has identified ß–lactam and macrolide resistance, the effects of widespread fluoroquinolone use in community acquired respiratory infections are largely not known. Therefore, to evaluate the *in vitro* activity of the new anti-gram positive quinolones particularly moxifloxacin in comparison to the currently available antibacterial agents, a UK wide sensitivity survey was performed. The sensitivity survey chose to focus on the three major respiratory pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. A total of 909 clinical isolates were collected from nine centres around the UK and sent to Edinburgh prior to sensitivity testing.

One of the advantages of this study was that organism identification and MIC determinations were undertaken by the same laboratory thereby removing the doubt that differences could be attributed to methodological variations and interpretation. However, a disadvantage is the nature of denominator data, which can introduce a bias, as the reasons for the sample being sent to the laboratory were not known.

Furthermore, the genetic relatedness of the clinical isolates and pertinent details such as previous antibiotic therapy and patient age were not provided. The lack of this information does compromise the true value of the results produced by the survey. Nevertheless, the sensitivity data do provide an insight into the efficacy of the newer fluoroquinolones as therapeutic agents against clinical respiratory isolates. The results obtained for this survey are presented in Chapter 3, Section 3.3 .

Against *S. pneumoniae* (n = 257), the ß-lactams (penicillin, cefotaxime) and clarithromycin were found to exhibit similar activity where 90% of the isolates were inhibited at 1mg/l. Similar *in vitro* activities for these antibiotics have been reported previously by Zhanel *et al* (2000). Generally, the *in vitro* activities of the ß-lactams (penicillins and cephalosporins) and macrolides (clarithromycin) are interlinked since surveillance reports have highlighted an increasing prevalence of pneumococcal strains that are cross resistant to both the ß-lactams and macrolides (Felmingham & Washington, 1999).

Against *S. pneumoniae* the rank order of activity was moxifloxacin > grepafloxacin > trovafloxacin = sparfloxacin > ciprofloxacin = levofloxacin. Ciprofloxacin and levofloxacin resistance have been reported in *S. pneumoniae* as both agents have not been found to be as active as the other new quinolones (Piddock *et al*, 1998) Tetracycline demonstrated the least activity against *S. pneumoniae* with an MIC_{90} of 32mg/l. Similar to the emergence of ß-lactam resistance, the increase in tetracycline resistance has been found to be proportional to its use in Spain (Baquero *et al*, 1991). The lack of such data within the UK does not allow comparisons between clinical usage of the drug and the elevated tetracycline MIC reported here.

The results presented here generally agree with previous sensitivity data, where the newer extended-spectrum quinolones exhibit improved activity against pneumococcal isolates (Woodcock *et al*, 1997; Dalhoff, 1999, Felmingham & Washington, 1999). The fluoroquinolones were found to be equally active against *S. pneumoniae* regardless of penicillin susceptibility. Generally, this observation is confirmed by Felmingham & Washington (1999) and Barry *et al* (1996), although some surveillance reports report a link between penicillin and quinolone resistance (Chen *et al*, 2000; Goldsmith *et al*, 1998). The association between penicillin and quinolone resistance has no theoretical basis, as it is well known that the mechanisms of resistance to both antibiotic classes are distinct. Surveillance studies have shown an increasing prevalence of macrolide and penicillin resistant pneumococcal strains, which evidently require other therapeutic options. Thus, it is possible that the link between penicillin and ciprofloxacin resistance is due to the increased use of quinolones against resistant pneumococcal isolates. In fact, two studies by Goldsmith *et al* (1998) and Chen *et al* (2000) have shown that the increased use of ciprofloxacin against resistant strains corresponded to the rise of penicillin, macrolide and quinolone resistant pneumococci. A further possibility is the clonal spread of ciprofloxacin resistant strains although there is no current data to support this.

Against gram-negative rods, all the fluoroquinolones, were found to be extremely active although ciprofloxacin and levofloxacin were less antibacterial than the other extended-spectrum fluoroquinolone agents tested. Against *H. influenzae*, levofloxacin was found to be less active than ciprofloxacin where 90% of the isolates were inhibited at 0.0625mg/l. Similar results were observed with *M. catarrhalis*, where 90% of the isolates were inhibited at 0.125–0.5mg/l to all the fluoroquinolones

with moxifloxacin, sparfloxacin, levofloxacin and ciprofloxacin being the most active. This result is concurred by (Felmingham & Washington, 1999; Woodcock *et al*, 1997). The tentative breakpoint recommendation for moxifloxacin versus *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* is ≥2mg/l for resistant and ≤1mg/l for sensitive strains (Andrews *et al*, 1999). All the respiratory pathogens tested exhibit sensitivity to moxifloxacin and were well within the recommended breakpoint value.

The major mechanism of resistance evident amongst clinical isolates of both *H. influenzae* and *M. catarrhalis* is ß-lactamase production. Data from the Alexander Project (Felmingham & Washington, 1999) and other studies (Doern *et al*, 1996) have shown that over 30% of *H. influenzae* and 90% of *M. catarrhalis* strains produce ß-lactamases which effectively renders the use of the aminopenicillins against these species futile. In the UK, the prevalence of amoxycillin resistance in *H. influenzae* has been reported at 14% and in this survey 8.2% of the isolates were found to be amoxycillin resistant based on the BSAC breakpoints. These results are similar to those reported by Richard *et al* (1998).

Against *M. catarrhalis*, amoxycillin was found to be the least active agent tested where 90% of isolates were inhibited at 16mg/l. None of the *M. catarrhalis* and *H. influenzae* isolates tested exhibited resistance to the amoxicillin/clavulanic acid combination (based on NCCLS breakpoint recommendations of 4mg/l) which concurs with the results of the Alexander Project data (Felmingham & Washington, 1999). Clarithromycin possessed poor activity against all three respiratory pathogens particularly against *H. influenzae* (8mg/l). Although, human volunteer studies on the

intrapulmonary distribution of clarithromycin demonstrated higher concentration of these agents in epithelial lining fluid than in serum (Rodvold *et al*, 1999) thereby, indicating that the MIC value for this agent may not correlate with its *in vivo* activity.

Generally, the MIC data reported for the fluoroquinolones against *H. influenzae* in this survey correspond with the values presented by Bruggemann *et al* (1997), which were four times lower than other sensitivity reports (Reinert *et al*, 1998). The similar values obtained for *H. influenzae* in this survey with those of Bruggemann *et al* (1997) can be explained by the similar incubation conditions employed. However, the values obtained for both *S. pneumoniae* and *M. catarrhalis* for moxifloxacin are four and two fold higher than that obtained by Bruggemann *et al* (1997), although the MIC data for both pathogens corresponds to the results of other investigators (Sahm *et al*, 2000; Bauernfeind, 1997; Andrews, 2000).

4.4 Structure-Activity Relationships

The fluoroquinolones studied here structurally differ at positions R_1, R_7 and X_8 (See Figure 1.1a). The sensitivity data presented here allows the analysis of specific structure-activity relationships of quinolone action against *S. pneumoniae*. The enhanced activity demonstrated by the anti-gram-positive quinolones like moxifloxacin, grepafloxacin, trovafloxacin and sparfloxacin in comparison to ciprofloxacin (4 to 8 times less active) were found to be restricted to structures that carry novel modifications at position C7.

Trovafloxacin and moxifloxacin possess an azabicyclo substituent, whereas grepafloxacin, sparfloxacin and levofloxacin retain a piperazine substituent, in

contrast to ciprofloxacin which only has an unsubstituted piperazine ring. Substituted piperazine-containing compounds have been shown to demonstrate greater activity against gram-positives due to the enhanced penetration potential into bacterial cells (Domagala, 1994). The methoxy group present at the C8 position in moxifloxacin seem to have no noticeable effect on antibacterial potency as fluoroquinolones that do not possess this modification (e.g. trovafloxacin) show equal activity against *S. pneumoniae*.

It was difficult to associate structural variations with enhanced antibacterial activity against both *H. influenzae* and *M. catarrhalis* as both organisms demonstrated similar MIC values to both the older agents and the extended-spectrum fluoroquinolones.

The unfortunate side effects associated with trovafloxacin, grepafloxacin and sparfloxacin and the subsequent formulary withdrawal of these agents precludes their use in the treatment of respiratory infections. Therefore, the widespread use of moxifloxacin in the treatment of these infections is to be expected.

4.5 Genetic Mutations within Type II Topoisomerases in *Streptococcus pneumoniae*: Contribution of Mutations to Fluoroquinolone Resistance

In this section, the contribution of individual genetic mutations within the type II topoisomerases will be analysed. The acquisition of quinolone resistance arises through mutations or amino acid changes within the quinolone resistance determining region (QRDR) within the subunits of DNA gyrase A (*gyrA*) and topoisomerase IV (*parC*). Recently, the cloning and analysis of *gyrB* (Pan & Fisher,

1999) and *parE* (Perichon *et al*, 1997) has identified the possible contribution of mutations within these genes in the development of quinolone resistance. In Chapter 3, the sequencing results of mutations in both subunits of topoisomerase IV and DNA gyrase A obtained from clinical isolates with reduced fluoroquinolone susceptibility and laboratory derived mutants challenged with different quinolones were presented. The initial aim of these experiments were to determine the intracellular targets of moxifloxacin in *S. pneumoniae* and to compare these mutational changes to those that occur when pneumococci are challenged by other quinolones. The mutational changes identified within the laboratory mutants were then compared against the changes sustained within the clinical population.

In *Streptococcus pneumoniae* resistance to the fluoroquinolones has been shown to develop in a stepwise manner through mutations within either of the target genes (DNA gyrase A and topoisomerase IV) or the hyperexpression efflux pumps (Hooper, 1998; Varon & Gutmann, 2000). The recent introduction of anti-gram-positive quinolones has prompted the investigation into the development of fluoroquinolone resistance when challenged with these new compounds. *In vitro* and *in vivo* studies identifying fluoroquinolone induced mutational changes in gram-negative bacteria have consistently shown that primary mutations occurs within *gyrA* (Tavio *et al*, 1999). In *S. pneumoniae,* it has been shown that ciprofloxacin challenge selects for mutations within topoisomerase IV (Pan & Fisher, 1996a). Ciprofloxacin challenge of other gram-positive bacteria like *S. aureus* also induce similar changes within *grlA* (*parC* equivalent in *S. aureus*) prior to changes within *gyrA* (Ferrero *et al*, 1995). These observations led to the assumption that the primary target of the fluoroquinolones in gram-positive bacteria is topoisomerase IV. However,

ciprofloxacin is a primarily anti-gram-negative agent unlike moxifloxacin, which is a broad-spectrum agent with preferential activity against gram-positive bacteria.

Therefore the question arises: do compounds with enhanced anti-gram positive activity select for different intracellular targets? To investigate this issue, stepwise selection of laboratory strain *S. pneumoniae* R6 with moxifloxacin was performed. Of the nine first-step mutants selected, two mutants 1TS7 and 1TS9 demonstrated a four-fold increase in the moxifloxacin MIC in comparison to the parent strain *S. pneumoniae* R6. Genetic analysis of 1TS7 and 1TS9 demonstrated the presence of a *gyrA* (Ser83 → Tyr) mutation within 1TS7 and no changes in 1TS9. Further selection of 1TS7 yielded second generation mutants that maintained the *gyrA* change and also acquired a *parC* change. Second-step mutants derived from 1TS9 did not develop any further mutations and did not exhibit further increases in MIC to any of the fluoroquinolones tested. Sensitivity testing of the first-step and second-step mutants derived from 1TS7 demonstrated that the acquisition of the *gyrA* mutation Ser83 → Tyr was able to increase the MIC to both ciprofloxacin and moxifloxacin MICs by 4-fold and eight-fold respectively. The subsequent acquisition of the *parC* mutation resulted in an 8–16 fold increase in the MICs to both ciprofloxacin and moxifloxacin. The differences observed in the MICs to both ciprofloxacin and moxifloxacin indicate that mutations within both *parC* and *gyrA* are able to mediate quinolone resistance but to varying levels. The initial mutation of Ser83 → Tyr within 1TS7 led to a higher increase in the MIC of moxifloxacin in comparison to ciprofloxacin. The increase in MIC observed with ciprofloxacin following the acquisition of the *gyrA* (Ser83 → Tyr) mutation suggests that

mutations within *parC* may not be the sole contributory factor to the decrease in sensitivity to ciprofloxacin.

Analysis of both the *parE* and *gyrB* genes indicated no changes within these genes, suggesting that moxifloxacin challenge does not select for changes within these genes and in this mutation study did not contribute to the decrease to both moxifloxacin and ciprofloxacin susceptibility as previously suggested (Pan & Fisher, 1996a). The results obtained with the *S. pneumoniae* R6 mutation study suggest that the intracellular target of moxifloxacin is *gyrA*.

In order to confirm that *gyrA* is the intracellular target of moxifloxacin in other pneumococcal isolates, two clinical pneumococcal isolates penicillin intermediate isolate 285 and penicillin resistant isolate 158 were used to generate quinolone resistant mutants.

Sequencing analysis of first-step mutants 285/3 and 285/7 generated from penicillin intermediate *S. pneumoniae* 285, were found not to sustain any changes within the target genes although a 2–4 fold increase to both ciprofloxacin and moxifloxacin was observed. The increase in MIC in the absence of target mutations within the target genes could possibly be attributed to an efflux mechanism.

The second-step mutants derived from 285/3 were found to develop a *gyrA* mutation (Ser83 → Tyr) which caused a four-fold increase to both ciprofloxacin and moxifloxacin. Third-step mutants were found to maintain the *gyrA* change with an additional mutation in *parC* (Ser80 → Phe). Sensitivity testing did confirm increases to moxifloxacin, gatifloxacin and ciprofloxacin. The resultant increases in the MIC were found to affect gatifloxacin and ciprofloxacin more than moxifloxacin.

With *S. pneumoniae* 158 both the first-step and second step mutant clones were found not to sustain any *gyrA* changes. Although second-step mutants demonstrated a four-fold increase in MIC to both moxifloxacin and ciprofloxacin. With both the 285 mutant series and the 158 mutant series, a change in *parE* (Val460 → Ile) was identified in the parent strain and all subsequent mutant clones derived from it. This substitution was not considered to contribute to quinolone resistance, as it was present in both the moxifloxacin sensitive parent strain *S. pneumoniae* 285 and *S. pneumoniae* 158. Furthermore, transformation experiments performed by Pestova *et al* (2000) have shown that the Val460 → Ile substitution does not contribute to fluoroquinolone resistance.

Moxifloxacin challenge of different pneumococcal isolates have consistently indicated the development of *gyrA* changes prior to any other genetic substitution within the target genes. Transformation studies with *S. pneumoniae* R6 done by Varon *et al* (1999) reported that dual mutations in both *gyr*A and *par*C were required prior to an increase in the moxifloxacin MIC thereby suggesting that both topoisomerases might function as simultaneous intracellular targets for moxifloxacin. This contrasts to our findings where *gyrA* mutations were always the first change to be characterised and the acquisition of the *gyrA* change in the mutants studied was always accompanied by a significant MIC increase to moxifloxacin and gatifloxacin compared with mutants that only sustain a *parC* change. Pestova *et al* (2000) reported similar findings where the acquisition of a *gyrA* change resulted in a four-fold increase to moxifloxacin MIC (2mg/l) in comparison to the presence of a *parC* change where the MIC of moxifloxacin remained at 0.25mg/l.

Recent surveys investigating decreased fluoroquinolone susceptibility in clinical pneumococcal isolates have indicated that the majority of clinical isolates express an efflux phenotype (Brenwald, *et al,* 1998; Morrissey *et al*, 1999). The new fluoroquinolone agents such as moxifloxacin are targeted towards this phenotype within the clinical population. Therefore, the impact of moxifloxacin challenge of *pmrA* efflux hyperexpresser P1Z1/IN27 was performed to ascertain the changes induced by moxifloxacin challenge against a strain already expressing an efflux phenotype. The stepwise selection of P1Z1/IN27 generated first-step mutants with a Ser83 → Phenyalanine mutation in *gyrA*. The acquisition of this change contributed to a 2–4 fold decrease in sensitivity to ciprofloxacin and moxifloxacin.

From these results, it is apparent that moxifloxacin challenge institutes changes within the *gyrA* gene regardless of the expression of other resistance mechanisms (See Table 4.1).

Study	Quinolone / Organism	Type II Topoisomerase Mutation			Primary Target
		First Step	Second Step	Third Step	
This Thesis	Moxifloxacin / *S. pneumoniae* R6	GyrA Ser81 → Tyr or Efflux	ParC Ser79 → Tyr	—	*gyrA*
This Thesis	Moxifloxacin / *S. pneumoniae* 285	—	GyrA Ser81→ Tyr or Efflux	ParC Ser79 → Phe	*gyrA*
This Thesis	Moxifloxacin / *S. pneumoniae* 158	Efflux	Efflux	—	—
This Thesis	Moxifloxacin / *S. pneumoniae* P1Z1/IN27	GyrA Ser81 → Phe or Efflux	—	—	*gyrA*
Pestova *et al*, 2000	Moxifloxacin / *S. pneumoniae* CP1000	GyrA Ser81 → Phe	ParC Ser79 → Tyr/Phe	—	*gyrA*
Pan & Fisher, 1997	Sparfloxacin / *S. pneumoniae* 7785	GyrA Ser81 → Phe/Tyr or Efflux	ParC Ser79 → Tyr/Phe or Asp83 → Asn	—	*gyrA*

Table 4.1: Summary of Results presented in Thesis and Relevant Published Data.

The different mutational studies performed here indicate a general trend where the primary mutation after moxifloxacin challenge occurs in *gyrA*. Laboratory strain *S. pneumoniae* R6 and *pmrA* efflux hyperexpresser P1Z1/IN27 were found to sustain a *gyrA* change within the first-step mutants. Penicillin intermediate clinical isolate *S. pneumoniae* 285 sustained a *gyrA* mutation within the second-step mutants. The sole exception to this pattern was demonstrated by the penicillin resistant clinical isolate *S. pneumoniae* 158 which sustained no changes within the QRDR of both *gyrA* and *parC*.

It is evident that resistance to moxifloxacin arises either through an initial mutation within *gyrA* or through the induction of an efflux pump or via mutations outwith the QRDR. It is also perhaps significant that the clinical strain *S. pneumoniae* 285 demonstrated the emergence of a *gyrA* change in the second-step mutants and *S. pneumoniae* 158 sustained no mutational changes within both the first and second generation mutants, which is in contrast with the resistance development observed within the first step mutants generated from the laboratory strains *S. pneumoniae* R6 and the *pmrA* efflux hyperexpresser P1Z1/IN27. This observation implies that fitter strains are able to stave off the emergence of genetic mutations through adaptation or by employing other mechanisms of resistance.

Generally, factors that contribute to the antibacterial potency of the fluoroquinolones are the kinetics of drug uptake and the ability to inhibit DNA gyrase or topoisomerase IV. The results presented in this thesis show that the extended-spectrum quinolones like moxifloxacin that primarily target *S. pneumoniae* mediate through DNA gyrase. The different antibacterial activity of both moxifloxacin and ciprofloxacin against *S. pneumoniae* suggest that the quinolones interact with the pneumococcal topoisomerases in a different manner.

To investigate, mutants were generated from both ciprofloxacin and norfloxacin challenge. Both ciprofloxacin and norfloxacin generated mutants that sustained mutations within topoisomerase IV. Sequencing analysis of these *parC* mutations indicated the change to be either Serine80 → Phenylalanine or Serine80 → Tyrosine (This thesis, Pan & Fisher, 1997; Fukuda & Hiramatsu, 1999). Sensitivity testing of

these characterised mutants demonstrated that the *parC* mutations had a minimal effect on the antibacterial activity of both moxifloxacin and gatifloxacin.

Studies by Pestova *et al* (2000) have also identified a similar effect attributing the enhanced antibacterial activity to the structural modifications in moxifloxacin and gatifloxacin which, indicate that the primacy of target mutations is dependent on the selecting agent. The selective preference of DNA gyrase A or topoisomerase IV by the fluoroquinolones appear to be dependent on the structural characteristics of the fluoroquinolone molecule.

Sequencing analysis of the QRDR of quinolone-resistant *S. pneumoniae* challenged with different quinolones has demonstrated a general trend. The first group of quinolones, whose prototypes are ciprofloxacin (Pan & Fisher, 1996a; This thesis) and norfloxacin (Fukuda & Hiramatsu, 1999; This thesis) select for *parC* or *parE* mutations, suggesting that these drugs act preferentially through topoisomerase IV *in vitro*, whereas a second group, typified by sparfloxacin (Pan & Fisher, 1997) gatifloxacin (Fukuda & Hiramatsu, 1999) and moxifloxacin (Pestova *et al*, 2000; This thesis) select for *gyrA* mutants *in vitro.* Clinafloxacin (Pan & Fisher, 1998) and gemifloxacin (Heaton *et al*, 2000) appear to act through both enzymes. The major structural variations sustained by all the quinolones are found in either the C7 or C8 position.

Recent studies by Alovera *et al* (2000) have also shown that substitutions at the C7 position within the quinolone pharmacore can markedly affect the potency of the quinolone and change the specificity target of the quinolone from topoisomerase IV and DNA gyrase in *S. pneumoniae*. Furthermore, studies involving the mammalian

topoisomerase II have shown that the inhibitory action of quinolones is dramatically increased when methyl groups are added to the C7 position, this observations suggesting that topoisomerase II directly interacts with the C7 substituent (Elsea *et al*, 1993; Gootz *et al*, 1994). Structural analysis of moxifloxacin and ciprofloxacin indicates that moxifloxacin possesses an azabicyclo modification at position 7 and in contrast ciprofloxacin has an unsubstituted piperazinyl ring. The *in vitro* mutation studies presented here involving both quinolones show a selective targeting of DNA gyrase by moxifloxacin and topoisomerase IV by ciprofloxacin. In the light of studies by Alovera *et al* (2000) and Gootz *et al* (1994), it is tempting to speculate that modifications within the C7 position are responsible for target preferences between these proteins. However, since the involvement of the other quinolone modifications have not been determined it is perhaps premature to attribute the preferential targeting of *gyrA* over *parC* to the C7 position only. Furthermore quinolones such as trovafloxacin which also possess modifications at position C7 mediate through topoisomerase IV instead of DNA gyrase A which questions the contributory role of substituents at position 7 (Gootz *et al*, 1996).

The results of the mutation studies have shown that substitutions within different genes i.e. DNA gyrase A or topoisomerase IV have variable effects on fluoroquinolone sensitivity. The results from the mutations involving *S. pneumoniae* R6, *S. pneumoniae* 285 and *S. pneumoniae* 158 mutants series have clearly shown that *gyrA* mutations affect the activities of both moxifloxacin and ciprofloxacin. The *parC* changes in both the ciprofloxacin and norfloxacin mutants have shown that these changes have a minimal effect on quinolones like moxifloxacin and gatifloxacin.

Fukuda and Hiramatsu (1998) have used these differences as a marker to determine the intracellular targets of the different quinolones. The results from their experiments showed that mutants could be classified into three types: mutants possessing single-point mutations within the QRDR of the *parC* gene and found to be cross resistant to the selecting agents (ciprofloxacin and levofloxacin) and not sparfloxacin and gatifloxacin; the second mutant type sustained point mutations within the QRDR of the *gyrA* gene where cross resistance was observed towards the selecting agents gatifloxacin and sparfloxacin but not towards the other quinolones; and the third type which sustain no mutations within the QRDR but are cross resistant to compounds like ethidium bromide and thus are categorised as efflux mutants.

Our findings are generally in agreement to that identified by Fukuda and Hiramatsu (1999). Although, the results presented in Chapter 3, Section 3.5 , indicates that the acquisition of a single *gyrA* mutation is able to confer increases to both the older and newer agents and *parC* changes are limited to instituting increases to agents such as norfloxacin and ciprofloxacin. Analysis of the first-step mutants with no target mutations but increased moxifloxacin MICs does suggest that these clones may be efflux mutants, although no concurrent increases to other efflux substrates like tetracycline and ethidium bromide were observed.

The fundamental question, however, is whether these results generated *in vitro* reflect the progression of resistance in clinical isolates. It is difficult to assess the *in vivo* emergence of resistance to any antibiotic as the acquisition of target mutations can be due to pre-selection by other agents of the same class or that clinical isolates

generally sustain multiple mutations. Genetic analysis of pneumococcal isolates with high-level resistance to the fluoroquinolones indicates the presence of at least one mutation within either DNA gyrase or topoisomerase IV (Jones *et al*, 2000; Bast *et al*, 2000a).

Sequencing analysis of clinical isolates indicates that low level resistance to ciprofloxacin typically arises following an amino acid substitution for Ser79 or Asp83 in ParC and that intermediate to high level resistance is achieved through subsequent mutations at Ser81 or Glu85 in GyrA (Janoir *et al*, 1996) where, the acquisition of these *gyrA* mutations in combination with a *parC* change is able to increase the MIC to both the older comparators like ciprofloxacin and the newer extended-spectrum quinolones like moxifloxacin (This thesis; Pestova *et al*, 2000). This observation supports our results, where *gyrA* mutations singly or in combination with *parC* changes have been shown to decrease sensitivity to all the fluoroquinolones tested. The vast majority of studies analysing the mutations within fluoroquinolone resistant isolates indicate that substitutions within *parC* occur more frequently than changes within *gyrA* which always develop in combination with *parC* changes (Pestova *et al*, 2000). However, recent data by Jones *et al* (2000) showed that of 69 pneumococcal isolates tested 5 isolates sustained single point mutations in *gyrA* and this one change was sufficient to induce resistance to both the extended-spectrum quinolones and older agents such as ciprofloxacin.

The *in vitro* studies presented in this thesis also indicate a similar role for *gyrA*. The appearance of sole *gyrA* mutations indicate that these mutations are stable *in vivo* and provide support for the results obtained in the *in vitro* mutation studies presented

previously. However, it has to be conceded that the number of clinical studies illustrating the role of single *gyrA* mutations are few and further data are required.

The cloning of *parE* and *gyrB* has identified other quinolone target sites within bacteria. Analysis of clinical isolates and laboratory mutants has identified a role for *parE* and *gyrB* changes in contributing to fluoroquinolone resistance, although we have not been able to assign a significance to either change in our studies. The only *parE* (Val460 → Ile) change identified here has been shown not to contribute to fluoroquinolone resistance (Pestova *et al*, 2000). Also, the majority of *in vitro* studies and genetic analysis of clinical isolates have been unable to identify mutations within *gyrB* that can be attributed to fluoroquinolone resistance (This thesis; Jones *et al* 2000; Janoir *et al*, 1996). The obvious rarity of *gyrB* and *parE* changes question the contribution of these changes to quinolone resistance and the stability of these mutations *in vivo*.

Current sequencing data of clinical isolates have shown a multitude of single amino acid changes in fluoroquinolone resistant pneumococci (Jones *et al*, 2000; Bast *et al*, 2000a), however the primary question is whether all mutations within the topoisomerases contribute to fluoroquinolone resistance. Generally, sequencing data obtained from clinical isolates are compared against standard NCTC strains available within the GenBank database. Unfortunately, these comparisons underscore the biovariation that exists when investigating a diverse collection of clinical isolates in contrast to studying the contributions of individual mutations within isogenic laboratory mutants. This observation is exemplified by the results that many isolates with significant mutation(s) exhibit MICs overlapping those for wild type isolates

(See Chapter 3, Table 3.7). This overlap is most apparent with MICs required by isolates possessing single alterations such as Lys137 → Asn and Asp83 → Asn in ParC or Val460 → Ile in ParE demonstrating the minimal impact of these mutations on susceptibility. Only, transformation studies or site-directed mutagenesis can ascertain the role of these changes in the development of fluoroquinolone resistance.

The mutations (Ser83 → Tyr/Phe in *gyrA* and Ser80 → Tyr/Phe in *parC*) identified in this thesis have been shown to directly affect the activities of the fluoroquinolones through transformation studies (Pestova *et al*, 2000; Janoir *et al*, 1996; Varon *et al*, 1999) and thus indicate that changes within *gyrA* and *parC* are predominant mutations that impact significantly in the development of fluoroquinolone resistance.

The data presented so far has elucidated the role of target mutations in fluoroquinolone resistance. The recent identification of the pneumococcal efflux pump *pmrA* suggests that this mechanism could contribute to fluoroquinolone resistance both in the absence and presence of target mutations. Recent studies have shown that efflux mediated resistance contributes to decreased fluoroquinolone susceptibility in 10–20% of clinical pneumococcal isolates (Brenwald *et al,* 1998; Morrissey *et al*, 1999) and has been shown to contribute to low levels of fluoroquinolone resistance in clinical isolates (Brenwald *et al,* 1998). In this study, the contribution of efflux pump expression in moxifloxacin challenged mutants and wild type clinical isolates was examined.

4.6 Contribution of Efflux pump expression to Fluoroquinolone Resistance in *S. pneumoniae*

To examine the contribution of efflux pumps a phenotypic method was employed using the efflux pump inhibitor reserpine. Initially, the MICs of all the mutants from the different mutation series were determined and the results are presented in Chapter 3, Section 3.6 . All the mutation series did not exhibit reserpine potentiated reduction with either moxifloxacin or gatifloxacin which is consistent with the observation that *pmrA* does not seem to have any effect on the newer generation quinolones such as grepafloxacin (Morrissey *et al*, 1999) and gemifloxacin (Heaton *et al*, 1999, Broskey *et al* 2000). Reductions in susceptibilities to norfloxacin and ciprofloxacin both hydrophilic quinolones were observed indicating that reserpine potentiation of the fluoroquinolones is only restricted to the older quinolone agents. The lack of reserpine potentiated reduction in susceptibility to moxifloxacin, also a hydrophilic quinolone (Piddock & Jin, 1999) suggests that either moxifloxacin is not a substrate of *pmrA* or is affected by other efflux proteins not inhibited by reserpine. Our results showed 2–4 fold reductions in the moxifloxacin MIC in four mutants derived from *S. pneumoniae* R6 which is in contrast to the reported substrate profile of *pmrA* (Brenwald *et al*, 1997). Although, a similar reserpine mediated reduction was observed with *S. aereus* when moxifloxacin and sparfloxacin was tested in combination with reserpine (Schmitz *et al*, 1998b). The data presented in this thesis and Schmitz *et al* (1998b) does suggest that in some strains moxifloxacin may be partly excreted by a reserpine sensitive efflux pump.

Therefore, to investigate moxifloxacin uptake into these mutants a fluorescence assay was performed. The uptake profile of mutants generated from P1Z1/IN27 was also

analysed. The first-step mutant 1TS9 which, sustained no *gyrA* or *parC* changes within the QRDR of either gene appeared to accumulate significantly higher levels of moxifloxacin than the other mutants. In contrast, 4TS67 which sustained mutations in *gyrA* and *parC* accumulated the least levels of moxifloxacin. No differences in moxifloxacin accumulation was observed with mutant 2TS35 which also possessed mutations in both *gyrA* and *parC*. The moxifloxacin accumulation profiles of the efflux mutant series exhibited dramatically decreased levels of uptake in comparison to the parent strain P1Z1/IN27. The trend that is apparent from both the accumulation assays indicated lower levels of moxifloxacin accumulation by mutants that were more resistant. This observation is concurred by the work done with ciprofloxacin resistant mutants which accumulate lesser quinolone as they become more resistant (Piddock *et al*, 1997; Zeller *et al*, 1997; Kaatz & Seo, 1995). Comparisons of the uptake assays between the *S. pneumoniae* R6 mutants and the P1Z1/IN27 mutant series indicate that the differences in the levels of accumulation are less apparent in the latter group. Some studies have suggested that the presence of a capsule can affect the uptake profiles of bacteria. In this case, *S. pneumoniae* R6 is a non capsulated strain and P1Z1/IN27 possesses a capsule. Capsules have been shown to generate a higher degree of non-specific binding of the antibiotics to surface components (Zeller *et al*, 1997), therefore creating a background and decreasing the apparent differences in quinolone uptake.

Therefore to further examine the possible role of *pmrA* expression northern blot analysis of both the *S. pneumoniae* R6 and P1Z1/IN27 derived mutants were conducted. RNA degradation issues prevented the gene expression analysis of the *S. pneumoniae* R6 mutant series. With the P1Z1/IN27 series, northern blot analysis

of parent P1Z1/IN27, first-step mutants (Eff10, Eff21 and Eff25) and second-step mutants (Eff49 and Eff50) was done. Increased transcription levels were obtained with P1Z1/IN27 and first-step mutants Eff10 and a very faint signal was obtained for Eff50. The RNA dot blot analysis was generally in agreement with the northern blot data but with an additional second-step (Eff 49) mutant yielding a positive signal. The analysis of the efflux mutants by MIC testing with the inhibitor failed to show an reserpine potentiated decrease in susceptibility to either ciprofloxacin and norfloxacin contrary to that demonstrated by the parent strain prior to moxifloxacin challenge. The combination of the MIC data and northern blot results indicates either a possible downregulation of the *pmrA* pump thereby explaining the altered substrate profile observed with the MIC data or the involvement of another *pmrA* like pump.

Earlier studies have indicated that the absence of reserpine inhibition of moxifloxacin disqualifies its role as a substrate of *pmrA* (Brenwald *et al,* 1997). Therefore, to investigate, the levels of *pmrA* expression were analysed after ciprofloxacin and moxifloxacin induction. In the RNA dot blot analysis all the parent strains tested (*S. pneumoniae* R6, *S. pneumoniae* P1Z1/IN27 and *S. pneumoniae* 285) showed positive results with ciprofloxacin and moxifloxacin induction. In contrast positive signals were only observed with the parent strains *S. pneumoniae* R6 and *S. pneumoniae* P1Z1/IN27 with the northern blot analysis. Due to the apparent discrepancies of these results, it is difficult to extract any firm conclusions from these experiments.

Given the ubiquity of efflux pumps on bacterial surfaces it is possible to suggest that another efflux pump which is similar to *pmrA* or *pmrA* itself is involved in the import

and export of other hydrophilic quinolones such as moxifloxacin. It may be possible to suggest that both ciprofloxacin and moxifloxacin induce the expression of *pmrA* or a *pmrA* like pump based on the MIC results and uptake assays. Mutations described within the promoter region of NorA and the efflux pump have been shown to alter the substrate profile of the drug (Kaatz & Seo, 1995). Genetic analysis of the *pmrA* gene in both laboratory generated mutants and clinical isolates demonstrated no mutations, as, the region analysed and verified by bidirectional sequencing is small. It is possible that changes within the promoter or regulator region could explain the altered substrate profile described here.

The results presented in this thesis clearly suggest that DNA gyrase is the primary target of moxifloxacin in laboratory generated mutants. The preferential targeting of DNA gyrase by moxifloxacin appears to be confined to the A subunit of the topoisomerase as no mutations were found within *gyrB*. The absence of target mutations in some of the first-step mutants despite MIC increases does suggest a role for efflux mutations, although the specific role of *pmrA* in moxifloxacin resistance or any other efflux pump could not be determined. The development of moxifloxacin resistance is represented in the diagram below (See Figure 4.1). Transformation studies analysing genetic mutations provide support that the mutations described here are clinically relevant, although it is conceded that the events that trigger *in vivo* emergence of resistance can differ significantly from *in vitro* environments.

The results presented here illustrate the possibility of spontaneous emergence of resistance to moxifloxacin and other agents. Generally, the emergence of antibiotic resistant mutants occurs at antibiotic concentrations that are not sufficiently

bactericidal to all the phenotypes present in a given bacterial population. Recent work by Dong *et al* (1999) have shown that the emergence of drug resistant mutants is curtailed if sufficiently high antibiotic concentrations are used. This concentration is known as the mutant prevention concentration which is determined by applying 10^{10} cells to agar plates containing various concentrations of quinolones, usually to a concentration above the MIC where no resistant mutants were recovered (See Figure 4.1). When a relationship between quinolone structure and resistance development was assessed, it was found that quinolones that possess the C8-methoxy lowered the MPC concentration as these agents were particularly bactericidal against first-step *gyrA* mutants (Zhao *et al*, 1997). In the clinical situation, the model proposed by Dong *et al* (1999) could provide an advantage (See Figure 4.1).

Generally antibiotic concentrations below the MPC concentration are likely to select for resistant mutants and concentrations that are above this value may be toxic to the patient. The MPC has been shown to block the growth of the most resistant single-step mutant (Dong *et al,* 1999) and for resistance to develop mutants must harbour two or more resistance mutations.

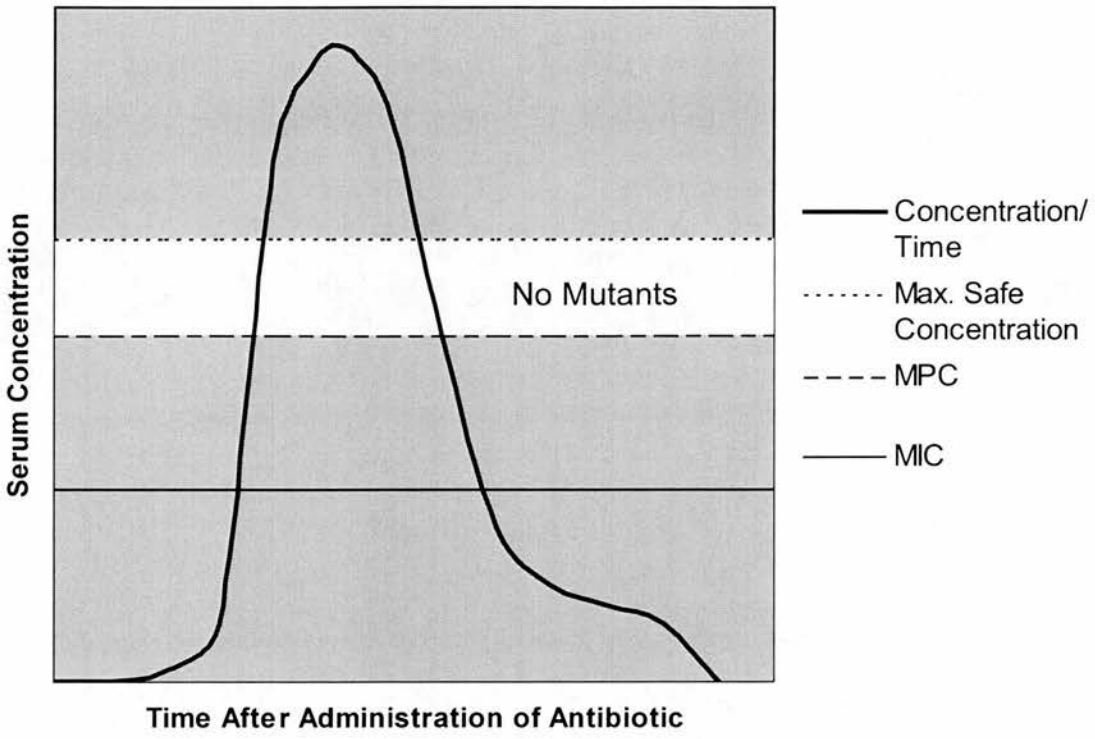


Figure 4.1: Mutant Selection Window

The development of double mutations in one mutation step is rare as has been exemplified by *in vitro* studies with both clinafloxacin (Pan & Fisher, 1998), gemifloxacin (Heaton *et al*, 2000) and moxifloxacin (This thesis, 2000). Thus, drug resistant mutations will be rare at the MPC prescribed antibiotic concentrations. It may be worthwhile to establish the use of MPC in drug dosages to prolong the use of potent antimicrobials and limit the *in vivo* emergence of resistant strains.

5 Conclusions

The sensitivity survey of respiratory clinical isolates has shown that fluoroquinolones particularly the extended-spectrum agents tested exhibit enhanced activity over the other clinically available options. In the face of ß-lactam and macrolide resistance, these agents provide an alternative, and the data presented here has shown the improved activity of these quinolones against previously resistant bacteria. It is conceded that the validity of these *in vitro* observations must await clinical studies after the widespread use of moxifloxacin.

The *in vitro* development of resistance to moxifloxacin in *S. pneumoniae* was found to arise through two pathways the first was the acquisition of a single *gyrA* mutation which resulted in an eight-fold increase in the moxifloxacin MIC and in the second pathway, regardless of the lack of target mutations an eight-fold increase in the MIC was observed suggesting that either the induction of an efflux pump or mutations

outwith the QRDR may be responsible. Mutation studies involving clinical pneumococcal isolates *S. pneumoniae* 285 and *S. pneumoniae* 158 reveal similar pathways of resistance development. Moxifloxacin selection of efflux hyperexpressing strain P1Z1/IN27 also demonstrated the development of a *gyrA* mutation prior to any other target changes. On the basis, of these results, it is proposed that moxifloxacin resistance arises initially through either an efflux mutation or by a *gyrA* change regardless of the strain phenotype.

In previous studies, it has been suggested that moxifloxacin is impervious to efflux mechanisms. The data presented on moxifloxacin accumulation and northern blot analysis indicates that efflux expression may play a role in the development of moxifloxacin resistance although the exact contribution was not determined. The *gyrA* and *parC* mutations identified in this study have been described in clinical pneumococcal isolates which is indicative of the stability and prevalence of these changes in *in vivo* situations. In this study no *gyrB* or *parE* mutations were identified after moxifloxacin challenge possibly indicating the rarity of these mutations and the secondary role they play in the development of resistance.

The target specificity of moxifloxacin differs from the older agents like ciprofloxacin and norfloxacin where topoisomerase IV changes have been shown to develop prior to any other target mutations. The topoisomerase IV mutations that develop after ciprofloxacin and norfloxacin challenge have been shown to have a minimal effect on the antibacterial activity of moxifloxacin. This observation was also confirmed by the enhanced activity of moxifloxacin against clinical pneumococcal populations which express an efflux mechanism and possess a target mutation.

Appendix I

Details of Strains Sent from Participating Centres

Centres	Number of Strains			
	H. influenzae	*S. pneumoniae*	*M. catarrhalis*	Total
Royal Infirmary, Aberdeen	—	—	48	48
Southmead Hospital, Bristol	32	26	40	98
St. James's Hospital, Dublin	37	17	14	68
Edinburgh Royal, Infirmary	74	64	39	177
Southern General, Glasgow	131	26	21	178
General Infirmary, Leeds	50	50	49	149
Royal Free, London	—	19	—	19
Hallamshire Hospital, Sheffield	43	18	12	73
University Hospital, Wales	32	37	30	99
Total	399	257	253	909

Appendix II

Minimum inhibitory concentrations and factor increases for moxifloxacin resistant mutants derived from *S. pneumoniae* R6.

Mutant Number	Moxifloxacin MIC (mg/l)	Factor Increase in Resistance
Parent Strain		
S. pneumoniae R6	0.0625	—
First Generation Mutants		
1TS1	0.0625	—
1TS2	0.125	2
1TS3	0.125	2
1TS4	0.125	2
1TS5	0.125	2
1TS6	0.0625	—
1TS7	0.5	8
1TS8	0.125	2
1TS9	0.5	8
1TS10	0.0625	—
1TS11	0.0625	—
Second Generation Mutants		
Derived from 1TS7		
2TS12	0.25	4
2TS13	0.25	4
2TS14	0.25	4
2TS15	0.25	4
2TS16	0.25	4
2TS17	0.25	4
2TS18	0.25	4
2TS19	0.5	4
2TS20	0.125	2
2TS22	0.5	2
2TS24	0.25	4
2TS25	0.25	4
2TS26	0.25	4

Mutant Number	Moxifloxacin MIC (mg/l)	Factor Increase in Resistance
2TS27	0.25	4
2TS28	0.25	4
2TS29	0.5	4
2TS30	0.25	4
2TS31	1	16
2TS32	0.25	4
2TS33	0.25	4
2TS34	0.25	4
2TS35	4	32
2TS36	2	32
2TS37	2	32
2TS38	2	32
2TS39	4	32
2TS40	2	32
2TS41	2	32
2TS42	2	32
2TS43	2	32
Derived from 1TS9		
2TS44	0.25	4
2TS45	0.25	4
2TS46	0.5	4
2TS47	0.5	4
2TS48	0.5	4
2TS49	0.125	2
2TS50	0.25	4
2TS51	0.25	4
2TS52	0.25	4
Third Generation Mutants		
3TS53	4	64
3TS54	4	32
3TS55	2	32
3TS56	2	32
3TS57	4	32
3TS58	4	64

Mutant Number	Moxifloxacin MIC (mg/l)	Factor Increase in Resistance
3TS59	8	64
Fourth Generation Mutants		
4TS63	2	32
4TS65	2	32
4TS66	4	64
4TS67	4	64
4TS68	8	64
4TS69	8	32
4TS70	8	128
4TS71	8	128
4TS72	8	128
4TS73	8	128
4TS74	4	64
4TS75	4	64
Fifth Generation Mutants		
5TS82	16	256
5TS86	16	256
5TS87	8	128
5TS88	8	128
5TS89	8	128
5TS90	8	128
5TS91	16	256

All MICs were determined by the doubling agar dilution method according to the BSAC guidelines (Phillips *et al*, 1991).
Mutants highlighted in bold are parent strains used to generate subsequent progeny.
All MIC increments are calculated in comparison to the original parent strain *S. pneumoniae* R6.

Appendix III

Minimum inhibitory concentrations and factor increases for moxifloxacin resistant mutants derived from *S. pneumoniae* R6.

Mutant Number	Moxifloxacin MIC (mg/l)	Change in Resistance
Parent Strain		
S. pneumoniae 285	0.0625	—
First Generation Mutants		
285/1	0.0625	—
285/2	0.0625	—
285/3	0.25	4
285/4	0.125	2
285/6	0.125	2
285/7	0.125	2
285/8	0.0625	—
285/9	0.125	2
285/10	0.25	4
285/11	0.125	2
285/12	0.0625	—
285/13	0.125	2
285/14	0.0625	—
285/15	0.125	2
285/16	0.0625	—
285/17	0.0625	—
285/18	0.125	2
285/19	0.125	2
285/20	0.125	2
Second Generation Mutants		
285/22	0.25	4
285/23	0.25	4
285/24	0.25	4
285/25	0.25	4
285/26	0.25	4
285/27	0.25	2

Mutant Number	Moxifloxacin MIC (mg/l)	Change in Resistance
285/28	0.25	2
285/29	0.25	2
285/30	2	32
285/31	2	32
285/32	1	16
285/33	1	16
285/34	1	16
285/35	1	16
285/36	1	16
285/37	1	16
285/38	1	16
285/39	1	16
285/40	1	16
285/41	1	16
285/42	1	16
285/43	1	16
285/44	1	16
285/45	1	16
285/46	1	16
285/47	1	16
285/48	1	16
285/49	1	16
285/50	1	16
285/51	1	16
285/52	1	16
285/53	1	16
285/54	1	16
285/55	1	16
Third Generation Mutants		
285/56	8	128
285/57	8	128
285/58	4	64
285/59	8	6
285/60	8	128

Mutant Number	Moxifloxacin MIC (mg/l)	Change in Resistance
285/61	8	128
285/62	4	64
285/63	4	64
285/64	4	64
285/65	8	128
285/66	8	128
285/67	4	64
285/68	4	64
285/69	4	64
285/70	4	64
285/71	4	64
285/72	8	128
285/73	4	64
285/74	4	64
285/75	8	128

All MICs were determined by the doubling agar dilution method according to the BSAC guidelines (Phillips *et al*, 1991).
Mutants highlighted in bold are parent strains used to generate subsequent progeny.
All MIC increments are calculated in comparison to the original parent strain *S. pneumoniae* 285.

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